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(54) METHOD FOR AMPLIFYING SPECIFIC NUCLEIC ACID SEQUENCES

VERFAHREN ZUR AMPLIFIZIERUNG SPEZIFISCHER NUKLEINSÄURESEQUENZEN

PROCEDE VISANT A AMPLIFIER DES SEQUENCES SPECIFIQUES D'ACIDES NUCLEIQUES

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- **HRUBAN R H ET AL: "K-RAS ONCOGENE ACTIVATION IN ADENOCARCINOMA OF THE HUMAN PANCREAS A STUDY OF 82 CARCINOMAS USING A COMBINATION OF MUTANT-ENRICHED POLYMERASE CHAIN RACTION ANALYSIS AND ALLELE-SPECIFIC OLIGONUCLEOTIDE HYBRIDIZATION" AMERICAN JOURNAL OF PATHOLOGY, vol. 143, no. 2, 1 August 1993 (1993-08-01), pages 545-554, XP000572114 ISSN: 0002-9440**
- **SHYR-YI LIN ET AL: "MUTATION ANALYSIS OF K-RAS ONCOGENES IN GASTROENTEROLIC CANCERS BY THE AMPLIFIED CREATED RESTRICTION SITES METHOD" AMERICAN JOURNAL OF CLINICAL PATHOLOGY, vol. 100, no. 6, 1 December 1993 (1993-12-01), pages 686-689, XP000572107 ISSN: 0002-9173**
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- **PROC. NATL. ACAD. SCI. U.S.A., Volume 89, (January 1992), WALKER G.T. et al., "Isothermal in Vitro Amplification of DNA by a Restriction Enzyme/DNA Polymerase System", pages 392-396.**

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Description

FIELD OF INVENTION

[0001] This invention relates to methods for *in vitro* amplification of specific nucleic acid target sequences. In particular the invention relates to methods which employ thermophilic restriction endonucleases to mediate selective amplification of nucleic acid targets which contain sequence differences including point mutations, deletions and insertions.

BACKGROUND OF THE INVENTION

[0002] A variety of inherited and acquired diseases are associated with genetic variations such as point mutations, deletions and insertions. Some of these variants are directly associated with the presence of disease, while others correlate with disease risk and/or prognosis. There are of the order of 500 human genetic diseases which result from mutations in single genes. These include cystic fibrosis, muscular dystrophy, α1-antitrypsin deficiency, phenylketonuria, sickle cell anaemia or trait, and various other haemoglobinopathies. Furthermore, individuals with increased susceptibility to several common polygenic conditions, such as atherosclerotic heart disease, have been shown to have an association with the inheritance of a particular DNA sequence polymorphism. Cancer is thought to develop due the accumulation of lesions in genes involved in cellular proliferation or differentiation. The *ras* proto-oncogenes, K-*ras*, N-*ras*, and H-*ras*, and the p53 tumour suppressor gene are examples of genes which are frequently mutated in human cancers. Specific mutations in these genes leads to activation or increased transforming potential. Genetic analysis is likely to become routine in the clinic for assessing disease risk, diagnosis of disease, predicting a patient's prognosis or response to therapy, and for monitoring a patient's progress. The introduction of such genetic tests depends on the development of simple, inexpensive, and rapid assays for genetic variations.

[0003] In rare instances mutations can be detected if they happen to lie within a naturally occurring restriction endonuclease recognition/cleavage site WO 84/01389 describes a method for discriminating between wild type genes and non wild type variants by screening for the presence or absence of restriction endonuclease sites. The inventors demonstrated the principle by analysis of variant sequences at codon 12 of the human H-*ras* proto-oncogene The wild type sequence at codon 12 forms part of the recognition/cleavage sites for the restriction endonucleases *Nae* I and *Hpa* II. Digestion with these endonucleases can discriminate between the wild type proto-oncogene and activated oncogenes which harbour mutations at this codon. Point mutations at codon 12 of H-*ras* are frequently found in bladder carcinomas and this general strategy could form the basis of screening kits for medical diagnosis.

[0004] Methods of *in vitro* nucleic acid amplification have wide-spread applications in genetics and disease diagnosis. The polymerase chain reaction (PCR) is a powerful, exquisitely sensitive procedure for *in vitro* amplification of specific segments of nucleic acids (R.K. Saiki, et al 1985 Science 230, 1350-1354 and F.F. Chehab, et al 1987 Nature 329, 293-294 and US 4683202 and US 4683195 and US 4800159 and US 4965188 and US 5176995). The PCR is mediated by oligonucleotide primers that flank the target sequence to be synthesized, and which are complementary to sequences that lie on opposite strands of the template DNA. The steps in the reaction occur as a result of temperature cycling (thermocycling). Template DNA is first denatured by heating, the reaction is then cooled to allow the primers to anneal to the target sequence, and then the primers are extended by DNA polymerase. The cycle of denaturation, annealing and DNA synthesis is repeated many times and the products of each round of amplification serve as templates for subsequent rounds. This process results in the exponential amplification of amplicons which incorporate the oligonucleotide primers at their 5' termini and which contain newly synthesized copies of the sequences located between the primers.

[0005] The PCR is extremely versatile and many modifications of the basic protocols have been developed Primers used for the PCR may be perfectly matched to the target sequence or they can contain mismatched and or modified bases. Additional sequences at the 5' end of primers can facilitate capture of PCR amplicons and the inclusion of labelled primers can facilitate detection. The inclusion of mismatched bases within primers can result in the induction of new restriction endonuclease recognition/cleavage sites. These sites can be located completely within the primer sequence. Alternatively, they can span a sequence which lies partially within the primer and partially within the newly synthesized target sequence (J.B. Cohen and A.D. Levinson (1988) Nature 334, 119-124). The general rules for designing primers which contain mismatched bases located near the 3' termini have been established (S. Kwok, et al. (1990) Nucleic Acids Research 18, 999-10005).

[0006] Modified primers containing mismatched bases were used to induce novel recognition/cleavage sites for restriction endonucleases in H-*ras* amplicons which were mutated at codon 12 (R. Kumar and M Barbacid (1988) Oncogene 3, 647-651) Similarly, primers containing mismatched bases were employed in protocols known as allele specific enrichment (Todd AV *et al* Leukemia, 1991, 5 160) or enriched PCR (Levi S *et al* Cancer Res., 1991, 6:1079) These are very sensitive protocols for the detection of point mutations. In these protocols, DNA samples were amplified with primers which induced either an *Eco* NI site in N-*ras* amplicons, or a *Bst* NI site in K-*ras* amplicons, provided the

sequences were wild type at codon 12. Aliquots of the PCR reactions were digested with the appropriate restriction endonuclease to cleave wild type amplicons prior to re-amplification of the digestion-resistant amplicons in a second round of the PCR. These protocols resulted in preferential amplification of sequences harbouring point mutations at codon 12 of *ras* More recently, a simplified enriched PCR protocol was published which allowed the reaction to be performed in a single tube (Singh *et al* Int J Oncol., 1994; 5: 1009). This protocol also required an initial round of PCR amplification, however, the restriction endonuclease was then added directly to the reaction tube. Following incubation with the restriction endonuclease, a second round of the PCR resulted in amplification of sequences harbouring mutations within the restriction endonuclease recognition/cleavage site. This analysis of natural or induced restriction endonuclease sites in PCR amplicons requires sequential activity of a DNA polymerase for the PCR, followed by activity of a restriction endonuclease for cleavage analysis. Enriched PCR protocols require sequential activity of firstly a DNA polymerase for the PCR, then restriction endonuclease activity to cleave specific sequences, followed by further DNA polymerase activity to re-amplify digestion resistant amplicons.

[0007] The ability to simultaneously exploit the activities of a restriction endonuclease and a DNA polymerase during the PCR could provide several advantages. It could allow the development of simple protocols for exclusive or preferential amplification of variant sequences in reactions which contain all reagents, including enzymes, at the initiation of the PCR. It was not previously known whether or not inclusion of a restriction endonuclease in a PCR could result in (i) complete (or partial) inhibition of amplification of a sequence which contains the recognition/cleavage site for the restriction endonuclease and (ii) exclusive (or preferential) amplification of a variant of this sequence which lacks the recognition/cleavage site for the restriction endonuclease. The ability to completely inhibit amplification of a sequence and/or exclusively amplify a variant sequence could lead to the development of protocols which do not require further manipulation prior to analysis. A reduction in the number of steps required for selective amplification and/or subsequent analysis of amplicons could lead to the development of protocols which are more rapid, less labour intensive and/or more amenable to automation. A further advantage is that reactions would be performed in a closed system and this would reduce the opportunity for contamination during the PCR

[0008] Such protocols would require concurrent activity of a restriction endonuclease and a DNA polymerase under conditions compatible with the PCR. The restriction endonuclease and the DNA polymerase must i) function in identical reaction conditions (eg., salt, pH) which must be compatible with the PCR and ii) must be sufficiently thermostable in these reaction conditions to retain activity during the thermocycling which is required for the PCR. Restriction endonucleases which are suitable for combination with the PCR must be active at temperatures which are compatible with stringent conditions for annealing of primers during the PCR, typically 50°C - 65°C. Simultaneous activity of thermophilic DNA polymerases and restriction endonucleases has previously been exploited to mediate *in vitro* amplification in an isothermal reaction known as strand displacement amplification (EP O 684 315 Al). It was not previously known whether restriction endonucleases could be sufficiently thermostable to maintain activity during the thermocycling required for the PCR.

SUMMARY OF INVENTION

[0009] In a first aspect the present invention consists in a method of detecting a genetic polymorphism in an individual, the method comprising the following steps:

(1) amplifying nucleic acid contained in a sample obtained from an individual by a process involving thermocycling of primers, the amplification occurring in the presence of a restriction endonuclease, the restriction endonuclease being sufficiently thermostable to retain activity during thermocycling, the primers being selected such that they introduce into either the nucleic acid amplified from the nucleic acid not including the polymorphism or from the nucleic acid including the polymorphism, a sequence recognised by he thermostable restriction endonuclease; and
(2) analysing the product of step (1) to determine the presence or absence of the polymorphism.

introduce the sequence recognised by the thermostable restriction endonuclease into the nucleic acid amplified from the nucleic acid not including the polymorphism.

[0010] In a second aspect the present invention consists in a method of detecting a genetic polymorphism in an individual, the method comprising the following steps:

(1) amplifying nucleic acid contained in a sample obtained from an individual by a process involving thermocycling of primers, the amplification occurring in the presence of a restriction endonuclease, the restriction endonuclease being sufficiently thermostable to retain activity during thermocycling, the restriction endonuclease being selected such that it recognises nucleic acid not including the polymorphism but not nucleic acid including the polymorphism or vice versa; and
(2) analysing the product of step (1) to determine the presence or absence of the polymorphism.

[0011] In one embodiment of this aspect of the present invention the thermostable restriction endonuclease recognises nucleic acid not including the polymorphism.

[0012] In a preferred embodiment of the present invention the method further comprises the following additional steps of:

(3) reacting the amplified nucleic acid from step (1) with at least one restriction endonuclease, the at least one restriction endonuclease being selected such that it digests the amplified nucleic acid including a particular polmorphism; and

(4) determining whether digestion occurs in step (3), digestion being indicative of the presence of the particular polymorphism.

[0013] There are a number of techniques for amplifying nucleic acid which involve thermocycling. These include polymerase chain reaction (PCR), ligase chain reaction, transcription-based amplification and restriction amplification. It is, however, presently preferred that the process involving thermocycling is PCR.

[0014] In yet a further preferred embodiment the step (2) analysis comprises detecting the presence or absence of amplified nucleic acid from step (1), the presence or absence of amplified nucleic acid indicating the presence or absence of the polymorphism.

[0015] Whilst the method of the present invention can be used with varying types of nucleic acid typically the nucleic acid will be DNA.

[0016] In yet another preferred embodiment of the present invention the thermostable restriction endonuclease is selected from the group consisting of *Bst* NI, *Bsl* I, *Tru* 9I and *Tsp* 509 I.

[0017] The method of the present invention can be used to detect a range of genetic polymorphisms including those occurring in one of the *ras* proto-oncogenes, K-*ras*, N-*ras*, and H-*ras*, or the p53 tumour suppressor gene, or in HIV-I, cystic fibrosis trans-membrane conductance regulator, α-antitrypsin or β-globin. The method of the present invention is particularly useful in detecting polymorphisms in codon 12 of K-*ras*.

[0018] The method of the present invention can be used for the analysis of a range of genetic polymorphisms including point mutations, small deletions and insertions. It was discovered that thermostable restriction endonucleases can be sufficiently thermostable to retain activity during thermocycling. It was also found that the PCR can be performed, using various polymerases, under the same buffer conditions which maintain activity and thermostability of the restriction endonucleases. It was discovered that the inclusion of a thermostable restriction endonuclease during the PCR can result in (i) inhibition of amplification of a sequence which contains the recognition/cleavage site for the restriction endonuclease and (ii) exclusive amplification of a variant of this sequence which lacks the recognition/cleavage site for the restriction endonuclease. These discoveries allowed the development of protocols for restriction endonuclease mediated selective PCR (REMS-PCR). REMS-PCR is simpler than other PCR protocols which utilise restriction endonucleases for the analysis of sequence variations. All components of the reaction are present at the initiation of the PCR and no subsequent manipulations are required prior to analysis. The reaction can therefore be performed in a closed vessel or chamber It was also found that the inclusion of a thermostable restriction endonuclease during PCR can result in (i) partial inhibition of amplification of nucleic acid which contains the recognition/cleavage site for the restriction endonuclease and (ii) preferential amplification of a variant of this sequence which lacks the recognition/cleavage site for the restriction endonuclease.

DETAILED DESCRIPTION OF THE INVENTION

[0019] As used herein, the following terms and phases are defined as follows:

[0020] The PCR is an *in vitro* DNA amplification procedure which requires two primers that flank the target sequence to be synthesized. A primer is an oligonucleotide sequence which is capable of hybridising in a sequence specific fashion to the target sequence and extending during the PCR. Amplicons or PCR products or PCR fragments are extension products which comprise the primer and the newly synthesized copies of the target sequences. Mulitplex PCR systems contain multiple sets of primers which result in simultaneous production of more than one amplicon. Primers may be perfectly matched to the target sequence or they may contain internal mismatched bases which can result in the induction of restriction endonuclease recognition/cleavage sites in specific target sequences. Primers may also contain additional sequences and/or modified or labelled nucleotides to facilitate capture or detection of amplicons. Repeated cycles of heat denaturation of the DNA, annealing of primers to their complementary sequences and extension of the annealed primers with DNA polymerase result in exponential amplification of the target sequence. The terms target or target sequence refer to nucleic acid sequences which are amplified The term template refers to the original nucleic acid which is to be amplified.

[0021] Restriction endonuclease mediated selective PCR (REMS-PCR) is an assay developed by the present inventor which applies the method of the present invention This assay requires simultaneous activity of a restriction

endonuclease and a DNA polymerase during the PCR. Restriction endonucleases which are suitable for REMS-PCR are preferably active at temperatures which are compatible with stringent conditions for annealing of oligonucleotide primers during the PCR, typically 50°C-65°C. A selection of commercially available restriction endonucleases which have high optimal incubation temperatures in this range are listed below in Table 1.

[0022] The term "individual" is used in herein in broadest sense and is intended to cover human and non-human animals, bacteria, yeast, fungi and viruses.

TABLE 1

Restriction Endonuclease	Recognition/Cleavage Sequence	Optimal Incubation Temperature (s)
Acc III	TCCGGA	65°C
Acs I/Apo I	(A/G)AATT(T/C)	50°C
Acy I	G(A/G)CG(C/T)C	50°C
Bco I	C(C/T)CG(A/G)G	65°C
Bsa BI/*Bsi* BI	GATNNNNATC	60°C/55°C
Bsa MI	GAATGCN	65°C
Bsa JI	CCNNGG	60°C
Bsa OI	CG(A/G)(T/C)CG	50°C
Bsa WI	(A/T)CCGG(A/T)	60°C
Bsc BI	GGNNCC	55°C
Bsc CI	GAATGCN	65°C
Bsc FI	GATC	55°C
Bse AI	TCCGGA	55°C
Bsi CI	TTCGAA	60°C
Bsi EI	CG(A/G)(C/T)CG	55°C
Bsi HKAI	G(A/T)GC(A/T)C	65°C
Bst LI	CC(A/T)GG	60°C
Bst MI	TCCGGA	60°C
Bst QI	TGATCA	60°C
Bst WI	CGTACG	55°C
Bst XI	ATCGAT	65°C
Bst ZI	GGNCC	60°C
Bsl I	CCNNNNNNNGG	55°C
Bsm I	GAATGCN	65°C
Bsm AI	$GTCTCN_1/N_5$	55°C
Bsm BI	$CGTCTCN_1/N_5$	55°C
Bss TII	CC(A/T)(A/T)GG	50°C
Bsr I	ACTGGN	65°C
Bsr DI	GCAATGNN	60°C
Bst 71I	$GCAGCN_8$	50°C
Bst BI	TTCGAA	65°C
Bst NI	CC(A/T)GG	60°C
Bst UI	CGCG	60°C
Bst YI	(A/G)GATC(C/T)	60°C
Bst ZI	CGGCCG	50°C
Dsa I	CC(A/G)(C/T)GG	55°C
Mae II	ACGT	55°C
Mae III	GTNAC	55°C
Mwo I	GCNNNNNNNGC	60°C
Ssp BI	TGTACA	50°C
Taq I	TCGA	65°C
Tfi I	GA(A/T)TC	65°C
Tru 9I	TTAA	65°C
Tsp 45 I	GT(C/G)AC	65°C
Tsp 509 I	AATT	65°C
Tsp R I	NNCAGTGNN	65°C
Tth 111 I	GACNNNGTC	65°C

A=adenine, G=guanine, T=thymine; C=cytosine, and N=A or G or T or C

[0023] In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described by reference to the following examples.

EXAMPLE 1

ASSAY FOR ASSESSING THE ACTIVITY/THERMOSTABILITY OF RESTRICTION ENDONUCLEASES

[0024] The activity/thermostability assay was used to examine the thermostability and residual enzymatic activity of restriction endonucleases including *Bst* NI, *Bsl I, Tru* 9I, and *Tsp* 509 I, in various buffer systems following a defined number of thermocycles.

[0025] The activity/thermostability of *Bst* NI, *Bsl* I and *Tru* 9I was compared for a variety of buffer conditions. Reactions contained primers (as indicated below in Table 2), each dNTP (dATP, dCTP, dTTP, dGTP) at 100 µM, 0.5 units of *Taq* DNA polymerase (5 units/µl; AmpliTaq®, Perkin Elmer) and either 20 units of *Bst* NI (10 units/µl; New England Biolabs) or *Tru* 9I (10 units/µl;Boehringer Mannheim) or 10 units of *Bsl* I (50 units/µl; New England Biolabs) in a total reaction volume of 25 µl.

Table 2

Primer	Amount (pmole)	Present in assay for	Sequence
5BKIT	7.5	*Bst* NI	TATAAACTTGTGGTAGTTGGACCT
5BKIQ	7.5	*Bsl* I, *Tru* 9I	TATAAACTTGTGGTACCTGGAGC
3KiE	7.5	*Bst* NI, *Bsl* I, *Tru* 9I	CTCATGAAAATGGTCAGAGAAACC
5BKIW	1.25	*Bsl* I	TTTTGTCGACGAATATGATCC

[0026] In addition, reactions contained one of the following basic buffer systems (set out in Table 3) with or without various additional reagents.

Table 3

Basic Buffer Name *** New England Biolabs ** Boehringer Mannhein * Perkin Elmer	Salt	Tris HCl (pH at 25°C)	$MgCl_2$ mM	DTT mM
*** NEB 2 **SuRE/Cut M	50 mM NaCl	10 mM (7.9)	10	1
*** NEB 3	100 mM NaCl	50 mM (7.9)	10	1
* PCR Buffer II	50 mM KCl	10 mM (8.3)		
* Stoffel Buffer	10 mM KCl	10 mM (8.3)		
MTris10	50 mM NaCl	10 mM (8.0 or 8.3 or 8.5 or 8.75)		
Htris50	100 mM NaCl	50 mM (8.0 or 8.3 or 8.5 or 8.75 or 9.0 or 9.5)		

[0027] The reactions were placed in a GeneAmp® PCR system 9600 thermocycler (Perkin Elmer), heated to high temperature and thermocycled as indicated in Table 4.

Table 4

Restriction Endonuclease	*Bst* NI	*Bsl*I	*Tru 9*I
Initial Temperature	94° C for 2 min.	92° C for 1 min.	94° C for 2 min.
Thermocycling	60° C for 1 min 92° C for 20 sec	55° C for 1 min 92° C for 20 sec	65° C for 1 min 92° C for 20 sec
Number of thermocycles	15 or 30	15 or 30	15 or 30

Table 4 (continued)

Optimal Incubation Temperature	60° C	55° C	65° C

[0028] Following thermocycling, 8 µg of plasmid DNA in a volume of 5 µl (pGFP-C1; Clontech) was added to each tube and the reactions were incubated for 1 hour at the optimal temperature as indicated by the manufacturer. The ability of the restriction endonuclease to cleave the plasmid DNA was assessed by electrophoresis on 3% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The endonuclease was scored as either being inactivated (I); having low (L), moderate (M) or high (H) activity; or having full (F) activity. (Table 5)

Table 5

Basic Buffer	Additional Reagents	*Bst* NI Activity		*Bsl* I Activity		*Tru* 9I Activity	
		Cycles		Cycles		Cycles	
		15	30	15	30	15	30
NEB2 SuRE Cut M		M	L			M	L
NEB3		F	M				
1 x PCR Buffer II	3 mM $MgCl_2$	M	L				
	6 mM $MgCl_2$	M	L	I	I		
	10 mM $MgCl_2$	H	M	I	I		
	10 mM $MgCl_2$: 1 mM DTT			H	M		
1 x Stoffel Buffer	3 mM $MgCl_2$	M	L				
	6 mM $MgCl_2$	M	L	I	I		
	10 mM $MgCl_2$	M	L	I	I		
	10 mM $MgCl_2$: 1 mM DTT			H	M		
MTris 10 pH 8.3 HTris 50 pH 8.3	10 mM $MgCl_2$	H	L	I	I		
		F	M				
MTris 10 pH 8.0	10 mM $MgCl_2$: 1 mM DTT						
MTris 10 pH 8.3	10 mM $MgCl_2$: 1 mM DTT			H	M		
MTris 10 pH 8.0	10 mM $MgCl_2$	M	L				
MTris 10 pH 8.3		H	L	I	I		
MTris 10 pH 8.5		M	L	I	I		
MTris 10 pH 8.75				I	I		
	10 mM $MgCl_2$; 1 mM DTT			H	M	H	L
HTris 50 pH 8.5	6 mM $MgCl_2$			I	I		
HTris 50 pH 8.5	6 mM $MgCl_2$: 1 mM DTT			H	M		
HTris 50 pH 8.0 HTris 50 pH 8.3 HTris 50 pH 8.5 HTris 50 pH 8.5	10 mM $MgCl_2$	F	M				
		F	M	I	I		
		F	M	I	I		
	10 mM $MgCl_2$: 1 mM DTT			H	M	H	L
HTris 50 pH 8.75	10 mM $MgCl_2$			I	I		
	10 mM $MgCl_2$; I mM DTT			H	M	H	L

Table 5 (continued)

Basic Buffer	Additional Reagents	*Bst* NI Activity		*Bsl* I Activity		*Tru* 9I Activity	
		Cycles		Cycles		Cycles	
		15	30	15	30	15	30
HTris 50 pH 8.3	3 mM $MgCl_2$	H	M				
	6 mM $MgCl_2$	F	M				
	10 mM $MgCl_2$	F	M	I	I		
HTris 50 pH 8.3	10 mM $MgCl_2$: 1 mM DTT			H	M		
HTris 50 pH 8.3 6 mM $MgCl_2$	-	F	M				
	1 mM DTT	H	M				
	0.1 mg/ml acetylated BSA (aBSA)	H	M				
	0.1 mg/ml non-acetylated BSA (non-a BSA)	H	M				
	1 mM DTT + aBSA	M	M				
	1 mM DTT + non-aBSA	M	L				
	10 % glycerol	H	M				
HTris50 pH 9.0	10 mM $MgCl_2$: 1 mM DTT					H	M
HTris50 pH 9.5	10 mM $MgCl_2$: 1 mM DTT					F	M

[0029] These experiments indicated that the activity/thermostability of restriction endonucleases during thermocycling varied considerably depending on both the restriction endonuclease and buffer system in which it was assayed. The pH and ionic strength of the Tris buffer, the choice and concentration of monovalent cation (K^+ or Na^+), the concentration of free Mg^{2+}, and the presence of other additives, particularly DTT, could influence the activity/thermostability. The influence of each of these components could depend on the other components in the buffer. For example, *Bst* NI retained more activity in PCR Buffer II containing 10 mM $MgCl_2$ than in this buffer containing either 3 or 6 mM $MgCl_2$. In contrast, varying the concentration of $MgCl_2$ between 3 and 10 mM had little effect on *Bst* NI activity when in HTris 50 (pH 8.3) buffer. In another example, the pH of the buffer had a greater influence on thermostability/activity of *Bst* NI in MTris 10 than in HTris 50.

[0030] *Bst* NI remains fully active following 15 thermocycles and moderately active following 30 thermocycles in buffer systems which contain either i) 100 mM NaCl, 50 mM Tris HCl (pH 8.3) and 6 mM $MgCl_2$ or ii) 100 mM NaCl, 50 mM Tris HCl (pH 8.0 - 8.5) and 10 mM $MgCl_2$ or iii) NEB 3 buffer and 0.1 mg/ml. *Bst* NI is more active in these buffers during thermocycling than in NEB 2 buffer with 0.1 mg/ml acetylated BSA which are the buffer conditions recommended by the manufacturers.

[0031] Similar experiments examining activity/thermostability of *Bsl* I indicted that this endonuclease requires the presence of 1 mM DTT in order to remain active following thermocycling. Provided DTT is present, *Bsl* I remains active in a broad range of conditions. *Bsl* I retains moderate activity following 30 thermocycles in buffer systems which contain i) PCR buffer II (Perkin Elmer), 1 mM DTT and 10 mM $MgCl_2$ ii) Stoffel buffer (Perkin Elmer), 1 mM DTT and 10 mM $MgCl_2$ iii) 50 mM NaCl, 10 mM Tris HCl (pH 8.5), 1 mM DTT and 10 mM $MgCl_2$ iv) 100 mM NaCl, 50 mM Tris HCl (pH 8.3 - 8.5), 1 mM DTT and 10 mM $MgCl_2$ or v) 100 mM NaCl, 50 mM Tris HCl (pH 8.5), 1 mM DTT and 6 mM $MgCl_2$. *Tru* 9I retains moderate activity following 30 thermocycles in a buffer system which contains 100 mM NaCl, 50 mM Tris HCl (pH 8.5 - 9.25), 10 mM $MgCl_2$ and 1 mM DTT. Experiments similar to those described above showed that *Tsp* 509 I retains moderate activity following 30 thermocycles in a buffer system which contains 50 mM NaCl, 10 mM Tris Hcl (pH 9 0 to 10), 10 mM $MgCl_2$ and 1mM DTT.

EXAMPLE 2

IDENTIFICATION OF BUFFER SYSTEMS COMPATIBLE WITH RESTRICTION ENDONUCLEASE AND DNA POLYMERASE THERMOSTABILITY/ACTIVITY AND THE PCR.

[0032] The range of buffers which was assessed for ability to maintain thermostable/activity of *Bst* NI (above) was

also assessed for compatibility with the PCR using primers 5BKIT or 5BKIW with 3KiE. The PCR mixtures containing genomic K562 DNA (800 ng), 30 pmole of 5BK1T or 30 pmole 5BKIW, 30 pmole of 3KiE, and each dNTP (dATP, dCTP, dTTP, dGTP) at 100 μM were set up for various buffer systems. Four units of *Taq* DNA polymerase (5 units/μl; AmpliTaq, Perkin Elmer) were mixed with TaqStart™ antibody (0.16 μl in 3.8 μl of antibody dilution buffer; Clontech) to give a final molar ratio of *Taq* DNA polymerase:TaqStart™ antibody of 1:5. The *Taq* DNA polymerase:TaqStart™ antibody mixture was incubated for 15 min at room temperature prior to addition to the mixtures. The total reaction volumes were 100 μl. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer), denatured at 94° C for 2 min and then subjected to 30 cycles of 60° C for 1 min followed by 92° C for 20 sec. Reactions were held at 60° C for 15 min after thermocycling.

[0033] A 28 μl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The gel was photographed using Stratagene Eagle Eye II video system. The efficiency of amplification with primers 5BKIT and 3KiE, or 5BKIW and 3KiE was rated as low, moderate or high. These primers were designed for use in a multiplex REMS-PCR system in conjunction with the restriction endonuclease *Bst* NI. The activity/thermostability assay on *Bst* NI and the PCR were performed in the same reaction buffers and subjected to the same thermocycling profile. The results of the two assay were examined to find conditions which allowed both efficient PCR amplification and preservation of restriction endonuclease activity. (Table 6)

Table 6

Basic Buffer	Additional Reagents	PCR Amplification Efficiency		*Bst* NI Activity	
		5BKIT 3KiE	5BKIW 3KiE	15 cycles	30 cycles
NEB2		High	High	Moderate	Low
NEB3		High	Moderate	Full	Moderate
PCR Buffer II	3 mM $MgCl_2$	High	High	Moderate	Low
	6 mM $MgCl_2$	High	High	Moderate	Low
	10 mM $MgCl_2$	High	High	High	Moderate
Stoffel Buffer	3 mM $MgCl_2$	High	High	Moderate	Low
	6 mM $MgCl_2$	High	High	Moderate	Low
	10 mM $MgCl_2$	Moderate	Moderate	Moderate	Low
MTris 10 pH 8.3	10 mM $MgCl_2$	High	High	High	Low
HTris 50 pH 8.3		Moderate	Moderate	Full	Moderate
MTris 10 pH 8.0	10 mM $MgCl_2$	High	Moderate	Moderate	Low
MTris 10 pH 8.3		High	High	High	Low
MTris 10 pH 8.5		High	Moderate	Moderate	Low
HTris 50 pH 8.0	10 mM $MgCl_2$	Moderate	Moderate	Full	Moderate
HTris 50 pH 8.3		Moderate	Moderate	Full	Moderate
HTris 50 pH *8.5*		Low	Moderate	Full	Moderate
HTris 50 pH 8.3	3 mM $MgCl_2$	High	Moderate	High	Moderate
	6 mM $MgCl_2$	High	Moderate	Full	Moderate
	10 mM $MgCl_2$	Moderate	Moderate	Full	Moderate

Table 6 (continued)

Basic Buffer	Additional Reagents	PCR Amplification Efficiency		*Bst* NI Activity	
HTris 50 pH 8.3 6 nM $MgCl_2$	-	High	Moderate	Full	Moderate
	DTT	High	Moderate	High	Moderate
	aBSA	High	Moderate	High	Moderate
	non-a BSA	High	Moderate	High	Moderate
	DTT + a BSA	High	Moderate	Moderate	Moderate
	DTT + non-a BSA	High	Moderate	Moderate	Low
	glycerol	Low	Low	High	Moderate
	T4 gene 32 protein	High	Low	Low	Inactive

[0034] The buffer conditions which simultaneously i) resulted in highly efficient amplification with the primer pair 5BKIT and 3KiE and moderately efficient amplification of the primer pair 5BKIW and 3KiE and ii) preserved full *Bst* NI activity for at least 15 thermocycles and moderate activity for 30 thermocycles, were selected for use in a REMS-PCR assay which requires concurrent activity of DNA *Taq* polymerase and *Bst* NI. Buffer conditions that fit these criteria were 100 mM NaCl, 50 mM Tris HCl pH 8.3 and 6 mM $MgCl_2$.

EXAMPLE 3

REMS-PCR USING *Bst*NI and DNA *Taq* POLYMERASE: ANALYSIS OF CODON 12 OF THE K-*RAS* GENE IN A MULTIPLEX SYSTEM INCORPORATING INTERNAL CONTROLS.

[0035] A REMS-PCR protocol was used to detect point mutations at codon 12 of the K-*ras* oncogene. The human cell lines Calu I [ATCC HTB54] and K562 [ATCC CCL243] were obtained from the American Type Culture Collection. Calu I is a lung adenocarcinoma cell which is heterozygous at K-*ras* codon 12 having both wild type (GGT) and mutant (TGT) sequences (D.J. Capon 1983 Nature 304, 507-513). K562 is a human leukemic cell line which is wild type at codon 12 of K-*ras* (R.L. Ward et al. Mol Pathol 1995 48, M273-277). Genomic DNA was extracted from Calu I and K562 by standard techniques (Sambrook et al 1989). DNA samples were amplified by REMS-PCR using primers 5BKIT, 5BKIW, 3MKiC and 3KiE. (Table 7)

Table 7

Primer	Function	Sequence
5BKIT	Diagnostic primer	TATAAACTTGTGGTAGTTGGACCT
5BKIW	PCR control primer	TTTTGTCGACGAATATGATCC
3MKiC	*Bst* NI control primer	CTGTATCAAAGCTTGGTCCTGGACCAG
3KiE	3' primer	CTCATGAAAATGGTCAGAGAAAC

[0036] The bold type C in the primer 5BKIT is mismatched with respect to the sequence of the K-*ras* gene. This mismatched base results in the induction of the recognition/cleavage site for *Bst* NI in K-*ras* amplicons provided that they are wild type at codon 12. Amplicons containing a mutation at either the first or second nucleotide of codon 12 do not contain the recognition/cleavage sequence for *Bst* NI. Primers 5BKIT and 5BKIW were biotin-labelled at their 5' ends and generate PCR amplicons which are similarly labelled. The bold type **G** in the primer 3MKiC is mismatched with the K-*ras* sequence. This results in the induction of a *Bst* NI recognition/cleavage site which is internal to the primer and which would be incorporated into any amplicons generated by amplification with this primer and either 5BKIT or 5BKIW.

[0037] Genomic DNA from K562, Calu I, and a 1:10 mixture (by weight) of Calu I:K562, was amplified in a multiplex REMS-PCR system. The reactions contained genomic DNA (800 ng), 30 pmole of 5BKIT, 30 pmole of 3KiE, 5 pmole of 5BKIW, 80 pmole of 3MKiC, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 μM, 80 units of *Bst* NI (10 units/μl, New England Biolabs) and 4 units of *Taq* DNA polymerase (5 units/μl; AmpliTaq, Perkin Elmer) in 100 mμM NaCl, 50 mM Tris (pH 8.3) and 6 mM $MgCl_2$. The total reaction volumes were 100 μl. Two control reactions contained either Calu I

DNA or dH_2O (no DNA) in the absence of *Bst*NI. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer), denatured at 94° C for 3 min and then subjected to 30 cycles of 60° C for I min followed by 92° C for 20 sec. Reactions were held at 60° C for 15 min following thermocycling.

[0038] A 25 µl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The gel was photographed using a polaroid land camera. In the control reaction, containing Calu I DNA in the absence of *Bst* NI, three fragments were clearly visible; a 185 bp fragment comprised of amplicons incorporating primers 5BKIT and 3KiE, a 156 bp fragment comprised of amplicons incorporating primers 5BKIT and 3MKiC, and a 114 bp fragment comprised of amplicons incorporating primers 5BKIW and 3KiE. A fragment of 85 bp comprised of amplicons incorporating primers 5BKIW and 3MKiC was faintly visible.

[0039] In the reactions containing *Bst* NI, the presence of the 185 bp fragment was diagnostic for the presence of K-*ras* codon 12 mutations. This fragment was visible in reactions containing Calu I and Calu I:K562 DNA at a ratio of 1:10, but not in reactions containing K562 DNA alone. The 156 bp (and 85 bp) *Bst* NI control fragments were not visible in any reactions containing *Bst* NI. This demonstrates that *Bst* NI can mediate complete inhibition of amplification of a second fragment. Since any 156 bp amplicon would contain a *Bst* NI site, inhibition of amplification of this fragment is not dependent on the mutational status of codon 12. Absence of restriction endonuclease control fragments allows unambiguous interpretation of negative results The 114 bp PCR control fragment was visible in all reactions including the reaction conlaining K562 DNA. This confirms that the conditions of the reactions, including the amount of template DNA, were adequate for amplification by the PCR. The presence of PCR control fragments allows unambiguous interpretation of positive results. No fragments were visible in the reaction containing no template DNA.

EXAMPLE 4

REMS-PCR: LIMIT OF DETECTION OF POINT MUTATIONS

[0040] The limits of detection of point mutations using REMS-PCR were assessed by analysing codon 12 of the K-*ras* gene in samples containing Calu I DNA diluted with Sup T1 DNA. Sup T1 [ATCC CRL 1942] is a leukemia cell line which was obtained from the American Type Culture Collection Calu I is heterozygous mutant at K-*ras* codon 12 and Sup T1 is wild type at codon 12 of the K-*ras* gene. Genomic DNA was extracted from these cell lines by standard techniques (Sambrook et al 1989) and amplified by the REMS-PCR. Calu I DNA was diluted with Sup T1 DNA at a ratio (by weight) of Calu I:Sup T1 of 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$ and $1:10^6$.

[0041] The REMS-PCR reactions contained genomic DNA (1 µg), 30 pmole of 5BK1T, 30 pmole of 3KiE, 5 pmole of 5BKIW, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 mM, and 40 units of *Bst* NI (10 units/µl, New England Biolabs) in 100 mM NaCl, 50 mM Tris (pH 8.3) and 6 mM $MgCl_2$. Four units of *Taq* DNA polymerase (5 units/µl; AmpliTaq, Perkin Elmer) were mixed with TaqStart™ antibody (0.16 µl in 3.8 µl of antibody dilution buffer; Clontech) to give a final molar ratio of *Taq* DNA polymerase:TaqStart™ antibody of 1:5. The *Taq* DNA polymerase:TaqStart™ antibody mixture was incubated for 15 min at room temperature prior to addition to the PCR mixture. The total reaction volumes were 100 µl. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer), denatured at 94° C for 2 min and then subjected to 30 cycles of 60° C for 1 min followed by 92° C for 20 sec. Reactions were held at 60° C for 15 min after thermocycling.

[0042] A 28 µl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The gel was photographed using a polaroid land camera and the Stratagene Eagle Eye II video system. The 185 bp fragment generated by amplification with primers 5BKIT and 3KiE was diagnostic for the presence of a mutation at codon 12. This fragment was visible in reactions containing Calu I:Sup T1 DNA at ratios of 1:10, $1:10^2$ and $1:10^3$ by polaroid photography and Eagle Eye imaging and in the reaction containing a ratio of $1:10^4$ by Eagle Eye imaging. This 185 bp fragment was not visible in the reactions containing Calu I:Sup T1 DNA at ratios of $1:10^5$ and $1:10^6$ nor in the reaction containing Sup T1 only. The 114 bp PCR control fragment generated by amplification with primers 5BKIW and 3KiE fragment was visible in all reactions. This confirms reaction conditions, including the amount of template DNA, were adequate for efficient amplification by the PCR.

[0043] The REMS-PCR reactions were also analysed in a colorimetric assay. This assay is similar to that described in Findlay et al (Clin. Chem. 1993 39/9, 1927-1933) PCR amplicons were specifically captured by hybridization to oligonucleotide probes that were covalently attached to latex beads which were applied at discrete locations in Periodontal Surecell blanks. The sequence of the capture oligonucleotides, and the specific PCR amplicons captured, are listed below (Table 8) K-Cap1 and K-Cap 2 were specifically designed to capture only diagnostic K-*ras* amplicons which were generated by amplification of mutant templates with the primers 5BKIT and 3KiE. K-Cap 3 is designed to capture amplicons generated by amplification of either mutant and wild type templates with either 5BKIT or 5BKIW and 3KiE. H-Cap 1 captures non-specific amplicons and provides a negative control for non-specific amplification or hybridization.

Table 8

Probe (Function)	Sequence	Sizes of fragments with homology (Primers incorporated)	Type(s) of amplicons captured
K-Cap 1 (Diagnostic)	TAGCTGTATCGTCAAGGCA CTCTT	185 bp (5BKIT/3KiE)	Mutant only
K-Cap 2 (Diagnostic)	AAATGATTCTGAATTAGCT GTATCGTC	185 bp (5BKIT/3KiE)	Mutant only
K-Cap 3 (PCR control)	GCACCAGTAATATGCATAT TAAAACAAG	185 bp (5BKIT/3KiE) 114 bp (5BKIW/3KiE)	Mutant Wild type
H-Cap1 (Negative control)	ACCATCCAGCTGATCCAGA ACCAT	Nil	Non-specific

[0044] Aliquots of the four oligonucleotide latex beads (0.25% in 1.6µl of 10mM Tris 1mM EDTA pH 7.4) were applied on to the Surecell membrane in discrete spots with all four oligonucleotides in each Surecell well. The oligonucleotide latex beads were allowed to dry for 15 minutes. Aliquots of 30 µl of each PCR was diluted with 170 µl of 50 mM KCl, 10 mM Tris (pH8.3) and 10 mM $MgCl_2$. The solution was denatured at 95° C for 6 min and applied to the Surecell well. The Surecells were then incubated at 50° C for 5 min to allow hybridization of PCR amplicons with the capture oligonucleotides. The wells were washed with 300 µl of 50 mM KCl, 10 mM Tris (pH 8.3) and 10 mM $MgCl_2$ at 50° C. The hybridized amplicons were reacted with three drops of a conjugate of streptavidin bound to horseradish peroxidase (EC 1.11.1.7) and incubated at room temperature for 2 min. The wash step was repeated to minimize non-specific interactions. Four drops of Leucodye/H_2O_2 were added and the Surecell were incubated at room temperature for 2 min. The immobilized complex served as a catalyst in the oxidative conversion of dye molecules from colourless to blue form The reaction was stopped with 4 drops of 0.1% NaN_3. The resultant coloured spots were scored visually by comparison against a colour chart and rated from 0 (no colour) to 10 (dark blue) (Table 9)

Table 9

Colour Score							
Calu 1:Sup T1 DNA	1:10	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$	Sup T1
K-Cap 1 (Mutant specific)	9	8	4	2	0	0	0
K-Cap 2 (Mutant specific)	9	8	4	2	0	0	0
K-Cap 3 (PCR Control)	9	9	9	9	9	9	9
H-Cap 1 (Non-specific negative control)	0	0	0	0	0	0	0

[0045] The sensitivity of the REMS-PCR protocol allowed detection of selectively amplified mutant sequences of K-*ras* codon 12 when present in a background of $1:10^3$ to $1:10^4$ wild type sequences when analysed by gel electrophoresis or a colorimetric assay. Wild type K-*ras* codon 12 sequences were not detected in this REMS-PCR assay. The literature suggests that this level of sensitivity will be adequate for analysis of DNA extracted from clinical specimens including tissue resections and biopsies, cytology samples and body fluids/excretions such as stools, urine and sputum containing small numbers of exfoliate tumour cells.

[0046] In a clinical setting, where large numbers of samples are simultaneously analysed, it is desirable that amplification does not commence prematurely as this can cause amplification of non-specific products including primer dimers. Monoclonal antibodies can bind to DNA *Taq* polymerase, and thus inhibit activity and amplification prior to the first denaturation step In initial experiments using REMS-PCR, the standard molar ratio of DNA *Taq* polymerase: TaqStart™ of 1:28, as recommended by Clontech, resulted in false positive results due to amplification of wild type Sup T1 DNA templates. Various molar ratios were tested and it was established that lower molar ratios of DNA *Taq* polymerase:TaqStart™ antibody such as 1:5 resulted in inhibition of non-specific amplification and primer dimer formation in the absence of false positive results.

EXAMPLE 5

ANALYSIS OF CLINICAL SPECIMENS USING REMS-PCR.

[0047] Genomic DNA was extracted by standard protocols (Sambrook et al 1989) from normal colon mucosa (NC) and colon adenocarcinomas (CA). Samples were analysed for the presence of K-*ras* codon 12 mutations by REMS-PCR as outlined in example 4 with the following protocol changes; DNA (0.5 μg) was amplified in the presence of 4 units of *Taq* DNA polymerase and 80 units of *Bst* NI. A 30 μl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD).

[0048] The 185 bp fragment generated by amplification with primers 5BKIT and 3KiE was diagnostic for the presence of a mutation at codon 12. This fragment was visible by gel electrophoresis in two reactions containing DNA from adenocarcinoma samples CA7 and CA8. This diagnostic fragment was not visible in two other reactions containing DNA from adenocarcinoma samples CA1 and CA2, nor in four reactions containing DNA extracted from normal colon mucosa; NC1, NC2, NC7 and NC8. The 114 bp control fragment generated by amplification with primers 5BKIW and 3KiE was visible in all reactions, indicating efficient PCR amplification had occurred in all reactions.

[0049] Genomic DNA from colon tissues had previously been analysed for the presence of mutations at K-*ras* codon 12 by standard enriched PCR (R.L. Ward et al Mol Pathol 1995 48, M273-277). Identical results were obtained when amplification was performed by either REMS-PCR or enriched PCR followed by analysis by gel electrophoresis. Both protocols indicated that DNA from adenocarcinoma samples CA7 and CA8 harboured mutations at K-*ras* codon 12 whereas the DNA from adenocarcinomas CA1 and CA2, as well as normal mucosa samples NC1, NC2, NC3 and NC4, were wild type at codon 12. These results demonstrate that REMS-PCR is suitable for rapid analysis of clinical specimens.

EXAMPLE 6: REMS-PCR: A SYSTEM WHICH ALLOWS IDENTIFICATION OF THE SPECIFIC NUCLEOTIDE SUBSTITUTION.

[0050] A REMS-PCR system was used to detect point mutations at codon 12 of the K-*ras* oncogene. Additional analysis with restriction endonucleases both confirmed the diagnosis of a mutation at codon 12 and allowed identification of the specific nucleotide substitution. The human cell lines Calu I [ATCC HTB54], A549 [ATCC], K562 [ATCC CCL243], Sup T1 [ATCC CRL 1942] and were obtained from the American Type Culture Collection. Calu I is a lung adenocarcinoma cell which is heterozygous at K-*ras* codon 12 having both wild type (GGT) and mutant (TGT) sequences (D.J. Capon 1983 Nature 304, 507-513). A549 is lung adenocarcinoma cell which is homozygous mutant (AGT) at K-*ras* codon 12 (D.M. Valenzuela and J. Groffen 1986 NAR 14, 843-852). K562 and Sup T1 are leukemic cell lines which are wild type at codon 12 of K-*ras*. Genomic DNA was extracted from these cell lines by standard techniques (Sambrook et al 1989).

[0051] REMS-PCR was performed with primers 5BKJT and 3AKIP which simultaneously induce multiple restriction endonuclease recognition/cleavage sites. Primers 5BK5 and 3K6 function as PCR control primers. (Table 10)

Table 10

Primer	Sequence : Bases mismatched with the K-*ras* gene which result in induction of restriction sites are indicated in bold type. (Additional mismatched bases are underlined)
5BKIT	TATAAACTTGTGGTAGTTGGACCT
3AKIP	GGATGACTCATTAAGGCACTCTTGCCTACGCCC
5BK5	TCAGCAAAGACAAGACAGGTA
3K6	AGCAATGCCCTCTCAAGA

[0052] The primer 5BKIT results in induction of a *Bst* NI recognition/cleavage site in K-*ras* amplicons which are wild type at codon 12. The primer 3AKIP induces one or more recognition/cleavage site(s) for the group of restriction endonucleases *Bsa* JI, *Sty* I, *Avr* II, *Mnl* I, *Aci* I, *Rle* I and *Bsu* 36I, in K-*ras* amplicons which are mutated at codon 12 as indicated below (Table 11).

Table 11

K-*ras* sequences and induced restriction endonuclease recognition/cleavage sites. (Mismatched bases introduced by 5BKIT (**C**) and 3AKIP (**G**) are indicated in bold type: Point mutations at codon 12 are underlined: N = T or A or C or G)				
	Codon 11	Codon 12	Codon 13	Restriction Endonuclease(s)
Wild Type sequence	CCT CCT	GGG GG	GGC	*Bst* NI
Mutant sequences	CCN	NGG		*Bsa* JI
	CCT	TGG		*Sty* I
	CCT	AGG		*Avr* II/*Sty* I
	CCT	C		*Mnl* I
		GCG	G	*Aci* I
	T	GTG	GG	*Rle* AI
	CCT	NAG GAG	G G	*Bsu* 36I *Mnl* I

[0053] The expected pattern of sensitivity and resistance of mutant amplicons to cleavage with the group of restriction endonucleases *Bsa* JI, *Sty* I, *Avr* II, *Mnl* I, *Aci* I, *Rle* I and *Bsu* 36I depends upon the exact mutation present at codon 12 and is indicated in Table 12.

Table 12

Codon 12 positions 1 and 2 (Point mutations are underlined; N = T or A or C)	Restriction endonucleases which cleave mutant amplicons	Restriction endonucleases which do not cleave mutant amplicons
NG	*Bsa* JI	
TG	*Bsa* JI/*Sty* I	*Avr* II
AG	*Bsa* JI/*Sty* I/*Avr* II	
CG	*Bsa* JI/*Mnl I*	*Bsu* 36I
GN		*Bsa* JI
GT	*Rle* AI	*Bsa* JI
GC	*Aci I*	*Bsa* JI
GA	*Mnl* I/*Bsu* 36I	*Bsa* JI

[0054] Genomic DNA from the human cell lines Calu I, A549, K562 and Sup T1 was amplified in a multiplex REMS-PCR system. The reactions contained genomic DNA (500 ng), 50 pmole of 5BKIT, 50 pmole of 3AKIP, 3 pmole of 5BK5, 3 pmole of 3K6, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 mM, 40 units of *Bst* NI (10 units/µl, New England Biolabs) in 100 mM NaCl, 50 mM Tris (pH 8.3) and 6 mM $MgCl_2$. Four units of *Taq* DNA polymerase (5 units/µl; AmpliTaq, Perkin Elmer) were mixed with TaqStart™ antibody (0.06 µl in 1.5 µl of antibody dilution buffer; Clontech) to give a final molar ratio of *Taq* DNA polymerase:TaqStart™ antibody of 1.2. The *Taq* DNA polymerase TaqStart™ antibody mixture was incubated for 15 min at room temperature prior to addition to the reactions. The total reaction volumes were 100 µl. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer), denatured at 94° C for 3 min and then subjected to 30 cycles of 60° C for 1 min followed by 92° C for 20 sec. Reactions were held at 60° C for 15 min after thermocycling.

[0055] A 20 µl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The gel was photographed using a polaroid land camera A 58 bp fragment, generated by amplification with primers 5BKIT and 3AKIP, was diagnostic for the presence of a mutation at codon 12. This fragment was visible in reactions containing Calu 1 and A549 DNA but was not visible in reactions

containing Sup T1 or K562 DNA A 167 bp PCR control fragment, generated by amplification with primers 5BK5 and 3K6 was present in all reactions, including reactions containing Sup T1 and K562 DNA. This confirmed that efficient PCR amplification had occurred in all reactions.

[0056] A 15 µl aliquot of the reactions containing Calu I or A549 DNA was digested with 10 units of the restriction endonucleases from the group *Bsa* JI, *Sty* I, *Avr* II, *Mnl* I, *Aci* I (as indicated below in Table 13) and incubated at the optimum temperature for digestion *as* specified by the manufacturer (New England Biolabs). The reactions were analysed by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD) and the gel was photographed using a polaroid land camera.

Table 13

Template DNA	Primers which generated amplicons	Restriction Endonuclease	Result	Sequence at codon 12 positions 1 and 2 (N = A, C or T)
K562	5K3/3K6 only	-	-	Wild type - GG
Sup T1	5K5/3K6 only	-	-	Wild type - GG
Calu I	5BKIT/3AKIP5K 5/3K6	*Bsa* JI *Sty* I *Avr* II	cleaves cleaves resistant	Mutant NG TG or AG not AG Result: Mutant (TG)
A549	5BKIT/3AKIP 5K5/3K6	*Bsa* JI *Sty* I *Avr* II	cleaves cleaves cleaves	Mutant NG AG or TG AG Result: Mutant (AG)

[0057] This REMS-PCR system allows detection of mutations at codon 12 of the K-*ras* oncogene. Subsequent analysis by restriction endonucleases confirms the presence of the mutation and allows identification of the specific nucleotide substitution.

EXAMPLE 7: REMS-PCR SYSTEM USING *Bst* NI AND STOFFEL POLYMERASE.

[0058] Genomic DNA from the human cell lines Calu I [ATCC HTB54] and Sup T1 [ATCC CRL 1942] was amplified by the REMS-PCR. Genomic DNA was extracted from these cell lines by standard techniques (Sambrook et al 1989) DNA was amplified by REMS-PCR in reactions containing genomic DNA (1 µg), 30 pmole of 5BKIT, 30 pmole of 3KiE, 2 pmole of 5BKIW, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 mM, and 40 units of *Bst* NI (10 units/µl, New England Biolabs) in 10 mM KCl, 10 mM Tris (pH 8.3) and 10 mM $MgCl_2$ (1 x Stoffel buffer, Perkin Elmer) A control reaction contained no DNA (dH_2O). Five units of Stoffel fragment (10 units/µl; Perkin Elmer) were mixed with Taq antibody TP4 (D.J, Sharkey et al 1994 Bio/technology 12, 506-509) (0.05 µl in 1.2 µl of Clontech antibody dilution buffer) to give a final molar ratio of Stoffel fragment: *Taq* antibody TP4 of 1:2. The Stoffel fragment: *Taq* antibody mixture was incubated for 15 min at room temperature prior to addition to the reactions. The total reaction volumes were 100 µl. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer), denatured at 94° C for 2 min and then subjected to 30 cycles of 60° C for 1 min followed by 92° C for 20 sec. Reactions were held at 60° C for 15 min after thermocycling.

[0059] A 25 µl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The gel was photographed using a polaroid land camera. The 185 bp fragment generated by amplification with primers 5BKIT and 3KiE was diagnostic for the presence of a mutation at codon 12. This fragment was visible in the reaction containing Calu I DNA, but was not visible in the reaction containing Sup T1 DNA. The 114 bp PCR control fragment, generated by amplification with primers 5BKIW and 3KiE, was visible in both reactions indicating efficient amplification by the PCR. No fragments were visible in the control reaction containing no template.

EXAMPLE 8: REMS-PCR SYSTEM USING *Bsl* I AND *Taq* DNA POLYMERASE.

[0060] A REMS-PCR assay was developed to detect point mutations at codon 12 of the K-*ras* oncogene. In this assay, amplicons contain the recognition/cleavage sequence for the thermophilic restriction endonuclease *Bsl* I pro-

vided they are wild type at codon 12. Amplicons which contain a mutation at either the first or second nucleotide of codon 12 do not contain the recognition/cleavage sequence for *Bsl* I.

[0061] Genomic DNA from the human cell lines Calu I [ATCC HTB54] and K562 [ATC CCL243]was amplified by the REMS-PCR. Calu I is heterozygous mutant at codon 12 of the K-*ras* gene and K562 is wild type at codon 12. Genomic DNA was extracted from these cell lines by standard techniques (Sambrook et al 1989). Calu 1 DNA was diluted with K562 DNA at a ratio (by weight) of Calu I:K562 of 1:10, $1:10^2$ and $1:10^3$

[0062] DNA was amplified by REMS-PCR using primers 5BKIQ, 5BKIW and 3KiH (Table 14). The 2 bold type C's in 5BKIQ are mismatched with respect to the sequence of the K-*ras* gene. These mismatched bases cause the induction of a *Bsl* I site in amplicons which are wild type at codon 12. Primers 5BKIQ and 5BKIW are biotinylated.

Table 14

Primer	Sequence
5BKIQ	TATAAACTTGTGGTACCTGGAGC
5BKIW	TTTTGTCGACGAATATGATCC
3KiH	GAAAATGGTCAGAGAAACC

[0063] The reactions contained genomic DNA (400 ng), 30 pmole of 5BK 1Q, 15 pmole of 3KiH, 0.5 pmole of 5BKIW, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 μM, and 10 units of *Bsl* I (50 units/μl, New England Biolabs) in 100 mM NaCl, 50 mM Tris (pH 8.5), 1 mM DTT and 6 mM $MgCl_2$. Eight units of *Taq* DNA polymerase (5 units/μl; AmpliTaq, Perkin Elmer) were mixed with TaqStart™ antibody (0.16 μl in 3.8 μl of antibody dilution buffer; Clontech) to give a final molar ratio of *Taq* DNA polymerase:TaqStart™ antibody of 1:5. The *Taq* DNA polymerase:TaqStart™ antibody mixture was incubated for 15 min at room temperature prior to addition to the reactions. The total reaction volumes were 50 μl. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer) and denatured at 94° C for 2 min. The reactions were then subjected to 10 cycles of 63° C for 30 sec followed by 92° C for 20 sec and then 20 cycles of 55° C for 1 min followed by 92° C for 20 sec. Reactions were held at 55° C for 15 min following thermocycling

[0064] A 28 μl aliquot of each reaction was analysed without subsequent manipulation by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD). The gel was photographed using a polaroid land camera. The 180 bp fragment generated by amplification with primers 5BKIQ and 3KiH was diagnostic for the presence of a mutation at codon 12. This fragment was visible in reactions containing Calu I:K562 at a ratio of 1:10 and $1:10^2$. This 180 bp diagnostic fragment was not visible in the reactions containing Calu I:Sup T1 at a ratio $1:10^3$ or in the reactions containing K562 only. The 109 bp PCR control fragment, generated by amplification with primers 5BKIW and 3KiH, was visible in all reactions indicating efficient amplification by the PCR.

[0065] This system utilized the restriction endonuclease *Bsl* I for detection of mutations at K-*ras* codon 12. This restriction endonuclease could be used in systems for the detection of mutations that occur at either codons 12 or 13 of any of the three *ras* oncogenes, K-*ras*, H-*ras* and N-*ras*. It could also be used for analysis of other mutations that occur in codons encoding either glycine or proline and for other mutations that occur at the nucleotides C or G.

EXAMPLE 9: ANALYSIS OF K-*ras* CODON 12 BY A REMS-PCR PROTOCOL WHICH REQUIRES SUBSEQUENT DIGESTION WITH *Bst* NI.

[0066] An alternative protocol was used to detect point mutations at codon 12 of the K-*ras* oncogene. Genomic DNA was extracted from Calu I [ATCC HTB54] and K562 [ATCC CCL243] by standard techniques (Sambrook et al 1989). Calu I DNA was diluted with K562 DNA at a ratio (by weight) of Calu I:K562 of 1:10, $1:10^2$, $1:10^3$ and $1:10^4$. DNA samples were amplified using primers 5BKIM which has the sequence GACTGAATATAAACTTGTGGTAGTTGGACCT and 3AKIL which has the sequence GGATGACTCATTTTCGTCCACAAAATGATTCTGAATTAG The bold type **C** in the primer 5BKIM is mismatched with respect to the sequence of the K-*ras* gene and results in the induction of the recognition/cleavage site for *Bst* NI in K-*ras* amplicons provided that they are wild type at codon 12. Bases within 3AKIL which are mismatched with K-*ras* are underlined.

[0067] The reactions contained genomic DNA (800 ng), 40 pmole of 5BKIM and 40 pmole of 3AKIL, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 μM, 10 μl of 10 X PCR Buffer II (Perkin Elmer), 1.5 mM $MgCl_2$, 80 units of *Bst* NI (10 units/μl, New England Biolabs) and 2 units of *Taq* DNA polymerase (5 units/μl; AmpliTaq, Perkin Elmer) in a total reaction volume of 100 μl. The reactions were placed in a GeneAmp PCR system 9600 (Perkin Elmer), denatured at 94° C for 3 min and then subjected to 40 cycles of 60° C for 1 min followed by 92° C for 20 sec. Reactions were held at 60° C for 15 min following thermocycling.

[0068] A 25 μl aliquot of each reaction was analysed without subsequent manipulation. A second 25 μl aliquot of

each reaction was incubated with 15 units of *Bst* NI (10 units/μl, New England Biolabs), 100 μg/ml bovine serum albumin (New England Biolabs) and 3.5 μl of 10 X NEB2 buffer (New England Biolabs) in a total reaction volume of 35 μl These reactions were overlayed with 20 μl of mineral oil and incubated overnight at 60°C All reactions were analysed by electrophoresis on a 5% Nusieve GTG gel (FMC Bioproducts, Rockland, MD) and photographed using a polaroid land camera.

[0069] In all reactions which had not been subjected to digestion with *Bst* NI following the PCR, a 103 bp fragment generated by amplification with primers 5BKIM and 3AKIL was visible. Following subsequent digestion with *Bst* NI, this 103 bp fragment was visible only in reactions containing Calu I:K562 DNA at ratios of 1:10, $1:10^2$ and $1:10^3$. The 103 bp fragment was not visible in the reactions containing Calu I:K562 DNA at a ratio of $1:10^4$ nor in the reaction containing K562 DNA alone. A 73 bp fragment, generated by *Bst* NI digestion of the wild type amplicons, was visible in all reactions. In reactions which were digested with *Bst* NI following the PCR, the presence of the 103 bp fragment was diagnostic for the presence of a mutation at codon 12 of K-*ras*.

[0070] The sensitivity of this protocol allowed detection of mutant Calu I DNA when present at a ratio of $1:10^3$ Calu I:K562 DNA. Under these reaction conditions, the inclusion of *Bst* NI in the PCR reaction resulted in preferential amplification (enrichment) of mutant sequences but did not result in complete inhibition of amplification of wild type K562 sequences. The reactions therefore required digestion with *Bst* NI prior to final analysis. Such protocols are of intermediate simplicity between standard enriched PCR protocols (which require two rounds of PCR plus an intermediate digestion with a restriction endonuclease to enrich for mutant sequences) and standard REMS-PCR protocols (where amplification of wild type sequences is completely inhibited and no subsequent manipulations such as digestion are required prior to analysis).

DISCUSSION

[0071] In REMS-PCR protocols the restriction endonuclease and the DNA polymerase must i) function in identical reaction conditions (eg., salt, pH) which must be compatible with the PCR and ii) be sufficiently thermostable in these reaction conditions to retain activity during the thermocycling which is required for the PCR. Some ofthe restriction endonucleases listed in Table 1, as well as other thermophilic restriction endonucleases, would be suitable for incorporation in REMS-PCR protocols provided buffer conditions can be identified which i) are compatible with restriction endonuclease activity and which maintain endonuclease activity while reactions are thermocycling during PCR and ii) are compatible with simultaneous DNA polymerase activity and which maintain polymerase activity while thermocycling during the PCR.

[0072] As little was previously known about the ability of restriction endonucleases to retain activity during the thermocyling required for the PCR, an assay which is simple and easy to conduct was developed to identify candidate thermophilic restriction endonucleases and reaction conditions. In the activity/thermostability assay, enzymatic activity of a restriction endonuclease, in a variety of reaction conditions, can be compared following a defined number of thermocycles. In this assay, reactions are prepared which contain primers, dNTPs, and DNA polymerase in concentrations which are standard for the PCR. The reactions contain no template DNA but include the buffer system, with or without additional reagents, and the restriction endonuclease to be examined. The reactions are placed on a thermocycler, subjected to a high temperature and then thermocycled. After a defined number of thermocycles reactions are removed, plasmid DNA is added to the tubes and the reactions are incubated at the optimal temperature for the restriction endonuclease as specified by the manufacturer. The enzymatic activity of the restriction endonuclease can be assessed by the degree of cleavage of the plasmid DNA as visualized by gel electrophoresis.

[0073] The activity/thermostability assay identified various restriction endonucleases, including *Bst* NI, *Bsl* I, *Tru* 9I and *Tsp* 509 I, which are sufficiently thermostable under certain buffer conditions to retain moderate or full catalytic activity following the thermocycling which is essential for the PCR. The reaction conditions which were most effective at preserving catalytic activity during thermocycling were identified. The catalytic activity of restriction endonucleases following thermocycling varied depending on the pH and ionic strength of the buffer, the choice and concentration of monovalent cation (K^+ or Na^+), the concentration of free Mg^{2+}, and the presence of other additives including dithiothreitol (DTT). The influence of each of these components can depend on the other components in the buffer.

[0074] It is also likely that the enzymatic activity of restriction endonucleases could be preserved by reducing the temperatures and times for DNA denaturation during the PCR. Factors known to influence the melting temperature of duplex DNA molecules include salt concentration, and the presence of reagents such formamide, dimethyl sulfoxide, glycerol and ethylene glycol. These reagents are compatible with at least some PCR systems. Inclusion of these, or other reagents which affect the DNA melting temperatures, may allow the PCR to be performed at decreased denaturation temperatures and/or times. These reagents may also have a direct positive or negative influence on the activity and/or thermostability of the restriction endonucleases (and/or DNA polymerase). The influence on the activity of restriction endonucleases of various thermocycling profiles, in the presence of additional reagents, can be assessed by the thermostability/activity assay described above. Identification of additional thermophilic restriction endonucleases,

and reaction conditions which preserve the activity of restriction endonucleases during thermocycling, can be achieved following routine testing using the activity/thermostability without the exercise of inventive skill

[0075] For REMS-PCR, the reaction conditions must not only preserve catalytic activity of the restriction endonucleases but they must also be suitable for the PCR. The buffer conditions must therefore be compatible with activity and thermostability of a DNA polymerase during thermocycling. There are many commercially available DNA polymerases which can be used for the PCR. These vary widely in their general properties, including both their optimal buffer conditions and the range of conditions they can tolerate. Examination of efficiencies of various DNA polymerases in the PCR, under reaction conditions which are known to preserve restriction endonucleases activity, allows identification of compatible DNA polymerase/restriction endonuclease/buffer combinations. A range of reaction conditions which had been demonstrated to maintain activity of restriction endonucleases, were also assessed for their compatibility with the PCR using various sets of primers and various DNA polymerases. The influence of different components of the reaction conditions on the PCR varied for different primer pairs and can depend on the other reaction components. For this reason, specific primers sets which are required for a PCR should be tested in this manner. Conditions for a PCR which are compatible with the concurrent activity of a restriction endonuclease and a DNA polymerase, and which result in efficient amplification with specific primer pairs can be identified following routine testing without the exercise of inventive skill.

[0076] REMS-PCR requires that the recognition/cleavage site for the thermophilic restriction endonuclease spans the nucleotide(s) which are to be analysed for genetic variations. This site can either occur naturally or may be induced by primers which contain internal mismatches to the template. When recognition/cleavage sites for restriction endonucleases are induced by primers. the sites lie partially within the primer and partially within the synthesized sequence which lies 3' to the primer in the amplicons. Primers must therefore include any mismatched bases which are required for induction of the restriction endonuciease site, but must not overlap the bases which are to be analysed. Rules for designing PCR primers which contain mismatched bases near the 3' terminus have been established (S. Kwok, et al. 1990. Nucleic Acids Research 18, 999-10005) While some terminally mismatched primers amplify inefficiently and reduce the yield of specific amplicons by up to 100 fold, the majority will amplify as efficiently as fully matched primers For example when the terminal 3' base in a primer is G it will extend on templates containing C, T or G, but not A, at the complementary position.

[0077] Recognition/cleavage sites can be more easily induced when the restriction endonuclease requires only a short tetranucleotide sequence for recognition (eg *Tru* 9I or *Tsp* 509 I) or when they recognise multiple sequences (eg *Bst* NI). Recognition/cleavage sites for restriction endonucleases which recognise short sequences which are interrupted are particularly amenable to induction. For example, *Bsl* I recognises the sequences CCNNNNNNNGG, where N is any nucleotide. *Bsl* I could be used to analyse mutations which occur at codons which encode either glycine (GGN) or proline (CCN). In general, primers designed to induce a *Bsl* I recognition site at these codons could be extended by DNA polymerases since they would not require mismatched bases near the 3' terminus and single or double mismatches located in the middle of a primer sequence are well tolerated and do not usually inhibit PCR amplification.

[0078] Furthermore, one skilled in the art could design primers capable of inducing a *Bsl* I recognition site for analysis of the vast majority (approximately 80%) of mutations that occur at either a G or a C. Mutation of the bases G and C are very common. For example, the percentage of p53 mutations that occur at either G or C residues is at least 77% of mutations in colorectal tumours, 72% of mutations in lung tumours, 74% of mutations in bladder tumours, 61% of mutations in breast tumours and 66% of mutations in brain tumours (M. Hollstein et al 1996 Nucleic Acids Research 24, 141-146). The following table lists all possible combination of sequences surrounding the bases C or G and the terminal bases which would be required for primers to induce CC or GG at these positions as part of the *Bsl* I site. The template/primer combinations which are predicted to be compatible with PCR are indicated in Table 15.

Table 15

Sequence of the template adjacent to the target base (underlined) N=A,C,G,T X=A,C,T Y=A,G,T	Primer Type Sense (5' primer) Anti-sense (3' primer)	Primer 3' base	Template 3' base	Compatible with PCR
G<u>G</u>N	Sense	G	C	Yes
A<u>G</u>N	Sense	G	T	Yes
C<u>G</u>N	Sense	G	G	Yes

Table 15 (continued)

Sequence of the template adjacent to the target base (underlined) N=A,C,G,T X=A,C,T Y=A,G,T	Primer Type Sense (5' primer) Anti-sense (3' primer)	Primer 3' base	Template 3' base	Compatible with PCR
TGX	Sense	G	A	No
NGG	Sense	N = as per template (sense)	N = as per template (anti-sense)	Yes
NCC	Anti-sense	G	C	Yes
NCT	Anti-sense	G	T	Yes
NCG	Anti-sense	G	G	Yes
YCA	Anti-sense	G	A	No
CCN	Anti-sense	N = as per template (anti-sense)	N = as per template (sense)	Yes

[0079] Examples of either natural or inducible recognition/cleavage sites for thermophilic restriction endonucleases in genes associated with acquired diseases are listed in Table 16. In these examples, restriction endonucleases which recognize wild type sequences are identified. The list includes restriction endonucleases which are known to be compatible with REMS-PCR and other endonucleases which are potentially compatible with the method. Primers for analysis of these mutations must include the bases which require induction (indicated in bold) but must not overlap the bases which are to be analysed (underlined). *Ras* proto-oncogenes (K-*ras*, H-*ras* and N-*ras*) are frequently activated in wide variety of human cancers by the acquisition of point mutations at codons 12, 13 and 61. Since codons 12 and 13 of all three *ras* genes code for glycine, *Bsl* I could be used for the analysis of the vast majority of *ras* mutations. A novel point mutation within intron D of H-*ras* has also been found in bladder carcinomas. Resistance of HIV strains to certain drugs is associated with the acquisition of point mutations.

Table 16

Gene	Disease	Cause	Wild Type Sequence (Bases to be analysed) Restriction endonuclease recognition site and name (Bases requiring induction)
K-*ras* N-*ras* H-*ras*	Cancer	Point mutations codons 12 and 13 eg K-*ras* codon 12 eg K-*ras* codon 13	GTTGGAGCTGG CCNNNNNNGG *Bsl* I GGAGCTGGTGG CCNNNNNNGG *Bsl* I
K-*ras*	Cancer	Point mutations codon 12	GCTGG CCTGG *Bst* NI
K-*ras* N-*ras* H-*ras*	Cancer	Point mutations codon 61-position 1 eg. H-*ras*	CCAGGAGGAGT CCNNNNNNNGG *Bsl* I
H-*Ras*	Cancer	Point mutations codon 61-position 3	CGCCGGCCAGG CCNNNNNNGG *Bsl* I
H-*ras*	Cancer	Point mutations codon 61 (except A to T at position 2)	CCAGG CCAGG *Bst* NI (CCTGG)
H-*ras*	Bladder Cancer	Point mutations Intron D	GTAA TTAA *Tru* 9I
HIV-I	AZT resistance	Point mutations 1. codon 41 2. codon 70 3. codon 215	1. GAAATG AATT *Tsp* 509I GCAATG *Bsr* DI 2. AAATGG AATT *Tsp* 509I 3. TTTACC TTAA *Tru* 9I
	ddI resistance	Point mutation codon 74	AAAATTA AATT *Tsp* 509I

[0080] A selection of genes which can harbour inheritable mutations associated with disease are listed in Table 17. The sequences listed are either wild type or mutant and the positions of potential sequence variations are underlined. Analysis of recessive mutations requires discrimination between heterozygous carriers and homozygous individuals with the latter at risk of disease development For all of the following examples, restriction endonuclease which would recognize the wild type sequences are identified For the cystic fibrosis transmembrane conductance gene, restriction endonucleases which recognize the mutated sequence have also been identified

Table 17

Gene	Disease	Sequence to be analysed	Type of Sequence/Sequence (Bases to be analysed) Endonuclease sites and names (Bases requiring induction)
Cystic fibrosis trans-membrane conductance regulator	Cystic fibrosis	Point mutations at 1. codon 542	Wild type sequence 1. ATAGTTCTTGG CCNNNNNNNGG *Bsl* I CCTGG *Bst* NI
		2. codon 551	2. CTGAGTGGAGGTCA CCNNNNNNNGG *Bsl* I GGTCC *Bsi* ZI
		3. IVS-4	3. TTATAAGAAGG CCNNNNNNNGG *Bsl* I
		4. Deletion codon 508 (3bp)	4. AAATATCATCTT GATNNNNATC *Bsa* BI *Bsi* BI
		Wild type sequences 1.codon 542	Mutant sequence 1. TCTTTGA TTAA *Tru* 9I
		2.codon 551	2. GATCAACGAG GATNNNNATC *Bsa* BI *Bsi* BI
		3.IVS-4	3. AAGAAGTTAA TTAA *Tru* 9I
		4. codon 508	4. AAATATCATTGG CCNNNNNNNGG *Bsl* I
α-antitrypsin	Emphysema Liver cirrosis	point mutation codon 342	Wild type sequence GACCATCGACG CCNNNNNNNGG *Bsl* I
β-globin	β-Thalassemia	Point mutation IVS-1 (β^o-Mediteranean)	Wild type sequence CCCTGGGCAGG CCNNNNNNNGG *Bsl* I
		Point mutation poly A signal (β^+-Black)	Wild type sequence AATAAA TTAA *Tru* 9I

[0081] Little was previously known about the effect of including a thermostable restriction endonuclease in a PCR. It was discovered that simultaneous activity of a restriction endonuclease and a DNA polymerase during the PCR can

result in (i) inhibition of amplification of a sequence which contains the recognition/cleavage site for the restriction endonuclease and (ii) selective amplification of a variant of this sequence which lacks the recognition/cleavage site for the restriction endonuclease. This discovery allows the development of protocols known as REMS-PCR. Such protocols could be used for the analysis of acquired or inherited polymorphisms, including point mutations, small deletions and insertions. When protocols for REMS-PCR are designed to detect mutant sequences, the wild type but not mutant sequences contain the recognition/cleavage sequence for a thermophilic restriction endonuclease. Amplification of wild type sequences by the PCR is inhibited by the activity of the restriction endonuclease. In contrast, mutant sequences are selectively amplified by DNA polymerase during the PCR.

[0082] Protocols for REMS-PCR can also be designed to selectively inhibit amplification of mutant but not wild type sequences. If protocols for REMS-PCR are designed to detect wild type sequences, the mutant but not wild type sequences contain the recognition/cleavage sequence for a thermophilic restriction endonuclease. Amplification of mutant sequences by the PCR would be inhibited by the activity of the restriction endonuclease and wild type sequences would be selectively amplified by the PCR. Failure to amplify specific wild type sequences would be consistent with a homozygous mutation. The ability to detect both wild type and mutant sequences would be consistent with the presence of a heterozygous mutation.

[0083] Several protocols for REMS-PCR were developed for the analysis of point mutations at codon 12 of the K-*ras* oncogene. These protocols exploited concurrent enzymatic activity of *Bst* NI and DNA *Taq* polymerase, or *Bst* NI and Stoffel fragment polymerase, or *Bsl* I and DNA *Taq* polymerase These protocols include multiplex primer systems which comprise diagnostic primers and one or two sets of control primers. The diagnostic primers induce a recognition/cleavage site for either *Bst* NI or *Bsl* I in K-*ras* amplicons provided positions 1 and 2 of codon 12 are wild type. Inclusion of one of these restriction endonucleases in the PCR results in inhibition of amplification of wild type DNA templates and selective amplification of DNA templates which contain mutations at positions 1 or 2 of codon 12. Amplification with these primers is therefore diagnostic for the presence of a point mutation at codon 12. Additional control primers are included in all reactions to confirm that the reaction conditions, including the amount of template DNA, are adequate for amplification by the PCR. These PCR control primers can flank any region which does not contain the endonuclease recognition/cleavage site. Amplicons incorporating these primers must be present for unambiguous interpretation of negative results. A second control primer was included in one multiplex system to confirm that the restriction endonuclease could mediate complete inhibition of amplification by the PCR. Control primers for the restriction endonucleases must either induce or flank the recognition/cleavage site for the restriction endonuclease used in the REMS-PCR protocol. Absence of amplicons incorporating these primers allows unambiguous interpretation of positive results.

[0084] The limits of detection of the REMS-PCR were assessed by analysis of samples containing Calu I DNA (heterozygous mutant at K-*ras* codon 12) diluted in Sup T1 DNA (wild type at K-*ras* codon 12) in the presence of *Bst* NI and DNA *Taq* polymerase The detection of diagnostic amplicons indicated the presence of K-*ras* sequences which were mutated at codon 12. Diagnostic amplicons were visualized, using gel electrophoresis and colorimetric analysis, in samples containing Calu I:Sup T1 at ratios of 1:10 to 1:10,000 but not in samples containing Sup T1 alone. PCR control amplicons were detected in all samples including Sup T1 DNA. The literature suggests that this level of sensitivity will be adequate for analysis of DNA extracted from clinical specimens including tissue resections and biopsies, cytology samples and body fluids/excretions such as stools, urine and sputum containing small numbers of exfoliate tumour cells (D.Sidransky et al. ,1992 Science 256, 102-1; L.Mao et al. 1994 Cancer Res. 54, 1634-1637). The application of REMS-PCR to the analysis of clinical specimens was demonstrated. Mutations at K-*ras* codon 12 were detected in DNA extracted from two out of four colon adenocarcinomas but none were detected in DNA extracted from four normal colon mucosas

[0085] In an extension of the REMS-PCR, the protocol can be performed with primers which simultaneously induce i) a recognition/cleavage site for a restriction endcnuclease that is present only in the wild type sequence and ii) multiple recognition/cleavage sites for restriction endonucleases that are specific for all possible mutated sequences. Subsequent analysis of diagnostic amplicons with the restriction endonucleases allows confirmation of the presence of a mutation in these amplicons and allows identification of the exact nucleotide substitutions in all cases.

[0086] It is also possible to develop REMS-PCR systems which result in selective amplification of mutant sequences but which do not result in complete inhibition of amplification of wild type sequences or *vice versa*. Reactions therefore require digestion with appropriate restriction endonuclease following PCR prior to analysis. Such protocols are of intermediate simplicity between standard enriched PCR protocols and standard REMS-PCR protocols where amplification of wild type sequences is completely inhibited.

[0087] REMS-PCR is compatible with a variety of capture and detection systems. This allows automation of the complete protocol and thus rapid analysis of large numbers of samples. Examples of capture systems include but are not restricted to i) PCR primers with a GCN4 recognition tag captured on GCN4 coated plates; ii) biotinylated primers captured with avidin or streptavidin; iii) digoxigenin-labelled products captured using anti-digoxigenin antibodies; and iv) complementary oligonucleotides attached to latex or magnetic beads. Examples of detection systems include, but are not restricted to, i) biotinylated PCR primers visualized with streptavidin/ horse radish peroxidase; ii) direct labelling

with molecules such fluorescein-isothiocyanate or alkaline phosphatase; and iii)-digoxigenin-labelled products detected using anti-digoxigenin antibodies:

[0088] REMS-PCR provides a sensitive, rapid method which is suitable for analysis of genetic variations which are associated with disease. The ability to simultaneously sustain the activities of a restriction endonuclease and a DNA polymerase during the PCR allows the development of simple protocols for selective amplification of variant sequences in reactions which contain all reagents, including all enzymes, at the initiation of the PCR. Reactions can be performed in a closed system which reduces the opportunity for contamination during the PCR. The protocol for REMS-PCR has fewer steps than other protocols which utilize restriction endonucleases to mediate selective amplification and/or analysis of mutant sequences. In general, the reactions do not require further manipulation prior to detection, however, the method does not preclude subsequent analysis of diagnostic amplicons for identification of the exact nucleotide substitution. A reduction in the number of steps required for selective amplification and analysis with restriction endonucleases makes the REMS-PCR assay rapid, less labour intensive and more amenable to automation.

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[0089]

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Claims

1. A method of detecting a genetic polymorphism in an individual, the method comprising the following steps:

   (1) amplifying the nucleic acid contained in a sample obtained from an individual by a process involving thermocycling of primers, the amplification occurring in the presence of a restriction endonuclease, the restriction endonuclease being sufficiently thermostable to retain activity during thermocycling, the primers being selected such that they introduce into either the nucleic acid amplified from the nucleic acid not including the polymorphism or from the nucleic acid including the polymorphism, a sequence recognised by the thermostable restriction endonuclease; and
   (2) analysing the product of step (1) to determine the presence or absence of the polymorphism.

2. A method as claimed in claim 1 in which the primers introduce the sequence recognised by the thermostable restriction endonuclease into the nucleic acid amplified from the nucleic acid not including the polymorphism.

3. A method of detecting a genetic polymorphism in an individual, the method comprising the following steps:

   (1) amplifying the nucleic acid contained in a sample obtained from an individual by a process involving thermocycling of primers, the amplification occurring in the presence of a restriction endonuclease, the restriction endonuclease being sufficiently thermostable to retain activity during thermocycling, the restriction endonuclease being selected such that it recognises nucleic acid not including the polymorphism but not nucleic acid including the polymorphism or vice versa; and
   (2) analysing the product of step (1) to determine the presence or absence of the polymorphism.

4. A method as claimed in claim 3 in which the thermostable restriction endonuclease recognises nucleic acid not including the polymorphism.

5. A method as claimed in claim 2 or claim 4 in which the method further comprises the following additional steps of:

   (3) reacting the amplified nucleic acid from step (1) with at least one restriction endonuclease, the at least one restriction endonuclease being selected such that it digests the amplified nucleic acid including a particular polymorphism; and

(4) determining whether digestion occurs in step (3), digestion being indicative of the presence of the particular polymorphism.

6. A method as claimed in any one of claims 1 to 5 in which the process involving thermocycling is PCR.

7. A method as claimed in any one of claims 1 to 6 in which the step (2) analysis comprises detecting the presence or absence of amplified nucleic acid from step (1), the presence or absence of amplified nucleic acid indicating the presence or absence of the polymorphism.

8. A method as claimed in any one of claims 1 to 7 in which the nucleic acid is DNA.

9. A method as claimed in any one of claims 1 to 8 in which the thermostable restriction endonuclease is selected from the group consisting of Bst NI, *Bsl* 1, Tru 91 and *Tsp* 509 I.

10. A method as claimed in any one of claims 1 to 9 in which the genetic polymorphism is detected in one of the *ras* proto-oncogenes, K-*ras*, N-*ras*, and H-*ras*, or the p53 tumour suppressor gene.

11. A method as claimed in claim 10 in which the genetic polymorphism is detected in codon 12 of K-*ras*.

12. A method as claimed in any one of claims 1 to 9 in which the genetic polymorphism is detected in HIV-I, cystic fibrosis trans-membrane conductance regulator, α-antitrypsin or β-globin.

13. A method as claimed in claim 9 in which the restriction endonuclease is *Bst* NI and the amplification of the nucleic acid occurs in the presence of a buffer including at least about 50mM NaCl and at least about 3mM $MgCl_2$.

14. A method as claimed in claim 13 in which the NaCl concentration is about 100mM.

15. A method as claimed in claim 13 in which the $MgCl_2$ concentration is 3 to 10mM.

16. A method as claimed in claim 9 in which the restriction endonuclease is *Bsl* I and the amplification of the nucleic acid occurs in the presence of a buffer including DTT.

Patentansprüche

1. Verfahren zum Detektieren einer genetischen Polymorphie bei einem Individuum, wobei das Verfahren folgende Schritte umfasst:

   (1) das Amplifizieren der Nucleinsäure, die in einer von einem Individuum erhaltenen Probe enthalten ist, durch ein Verfahren, das thermisches Zyklisieren von Primern umfasst, wobei die Amplifikation in Gegenwart einer Restriktionsendonuclease erfolgt, wobei die Restriktionsendonuclease ausreichend thermostabil ist, um während des Thermozyklus ihre Aktivität beizubehalten, wobei die Primer so gewählt sind, dass sie entweder in die Nucleinsäure, die aus der Nucleinsäure amplifiziert ist, welche die Polymorphie nicht umfasst, oder in die, die aus der Nucleinsäure amplifiziert ist, welche die Polymorphie umfasst, eine Sequenz einführen, die von der thermostabilen Restriktionsendonuclease erkannt wird; und
   (2) das Analysieren des Produkts aus Schritt (1), um das Vorhandensein oder Fehlen der Polymorphie zu bestimmen.

2. Verfahren nach Anspruch 1, bei dem die Primer die Sequenz, die von der thermostabilen Restriktionsendonuclease erkannt wird, in die Nucleinsäure einführen, die aus der Nucleinsäure amplifiziert wird, welche die Polymorphie nicht umfasst.

3. Verfahren zum Detektieren einer genetischen Polymorphie bei einem Individuum, wobei das Verfahren folgende Schritte umfasst:

   (1) das Amplifizieren der Nucleinsäure, die in einer von einem Individuum erhaltenen Probe enthalten ist, durch ein Verfahren, das thermisches Zyklisieren von Primern umfasst, wobei die Amplifikation in Gegenwart einer Restriktionsendonuclease erfolgt, wobei die Restriktionsendonuclease ausreichend thermostabil ist, um

während des Thermozyklus ihre Aktivität beizubehalten, wobei die Restriktionsendonuclease so gewählt ist, dass sie Nucleinsäure erkennt, welche die Polymorphie nicht umfasst, nicht aber Nucleinsäure, welche die Polymorphie umfasst, oder umgekehrt; und
(2) das Analysieren des Produkts aus Schritt (1), um das Vorhandensein oder Fehlen der Polymorphie zu bestimmen.

4. Verfahren nach Anspruch 3, bei dem die thermostabile Restriktionsendonuclease Nucleinsäure erkennt, welche die Polymorphie nicht umfasst.

5. Verfahren nach Anspruch 2 oder 4, bei dem das Verfahren weiters die folgenden zusätzlichen Schritte umfasst:

(3) das Umsetzen der amplifizierten Nucleinsäure aus Schritt (1) mit zumindest einer Restriktionsendonuclease, wobei die zumindest eine Restriktionsendonuclease so gewählt ist, dass sie die amplifizierte Nucleinsäure verdaut, die eine bestimmte Polymorphie umfasst; und
(4) das Bestimmen, ob in Schritt (3) Verdau erfolgt, wobei der Verdau ein Indikator für das Vorhandensein der bestimmten Polymorphie ist.

6. Verfahren nach einem der Ansprüche 1 bis 5, bei dem das Verfahren, das thermisches Zyklisieren umfasst, PCR ist.

7. Verfahren nach einem der Ansprüche 1 bis 6, bei dem die Analyse in Schritt (2) das Detektieren des Vorhandenseins oder Fehlens von amplifizierter Nucleinsäure aus Schritt (1) umfasst, wobei das Vorhandensein oder Fehlen von amplifizierter Nucleinsäure das Vorhandensein oder Fehlen der Polymorphie anzeigt.

8. Verfahren nach einem der Ansprüche 1 bis 7, bei dem die Nucleinsäure DNA ist.

9. Verfahren nach einem der Ansprüche 1 bis 8, bei dem die thermostabile Restriktionsendonuclease aus der aus Bst NI, Bsl 1, Tru 91 und Tsp 509 I bestehenden Gruppe ausgewählt ist.

10. Verfahren nach einem der Ansprüche 1 bis 9, bei dem die genetische Polymorphie in einem der ras-Proto-Onkogene, K-ras, N-ras und H-ras, oder im p53-Tumorsuppressorgen detektiert wird.

11. Verfahren nach Anspruch 10, bei dem die genetische Polymorphie in Codon 12 von K-ras detektiert wird.

12. Verfahren nach einem der Ansprüche 1 bis 9, bei dem die genetische Polymorphie in HIV-I, Mukoviszidose-Transmembran-Konduktanzregulator, α-Antitrypsin oder β-Globin detektiert wird.

13. Verfahren nach Anspruch 9, bei dem die Restriktionsendonuclease Bst NI ist und die Amplifikation der Nucleinsäure in Gegenwart eines Puffers erfolgt, der zumindest etwa 50 mM NaCl und zumindest etwa 3 mM $MgCl_2$ enthält.

14. Verfahren nach Anspruch 13, bei dem die NaCl-Konzentration etwa 100 mM beträgt.

15. Verfahren nach Anspruch 13, bei dem die $MgCl_2$-Konzentration 3 bis 10 mM beträgt.

16. Verfahren nach Anspruch 9, bei dem die Restriktionsendonuclease BstI I ist und die Amplifikation der Nucleinsäure in Gegenwart eines Puffers erfolgt, der DTT umfasst.

Revendications

1. Méthode de détection d'un polymorphisme génétique chez un individu, la méthode comprenant les étapes suivantes :

(1) amplifier l'acide nucléique contenu dans un échantillon obtenu d'un individu par un procédé impliquant le thermocycle des amorces, l'amplification se produisant en présence d'une endonucléase de restriction, l'endonucléase de restriction étant suffisamment thermostable pour conserver son activité pendant le thermocycle, les amorces étant sélectionnées de façon qu'elles introduisent dans l'acide nucléique amplifié à partir de l'acide nucléique ne comprenant pas le polymorphisme ou de l'acide nucléique comprenant le polymorphisme, une séquence reconnue par l'endonucléase de restriction thermostable ; et

(2) analyser le produit de l'étape (1) pour déterminer la présence ou l'absence du polymorphisme.

2. Méthode selon la revendication 1 dans laquelle les amorces introduisent la séquence reconnue par l'endonucléase de restriction thermostable dans l'acide nucléique amplifié à partir de l'acide nucléique ne comprenant pas le polymorphisme.

3. Méthode de détection d'un polymorphisme génétique chez un individu, la méthode comprenant les étapes suivantes :

(1) amplifier l'acide nucléique contenu dans un échantillon obtenu d'un individu par un procédé impliquant le thermocycle des amorces, l'amplification se produisant en présence d'une endonucléase de restriction, l'endonucléase de restriction étant suffisamment thermostable pour conserver son activité pendant le thermocycle, l'endonucléase de restriction étant sélectionnée de façon qu'elle reconnaisse l'acide nucléique ne comprenant pas le polymorphisme mais pas l'acide nucléique comprenant le polymorphisme ou vice versa ; et
(2) analyser le produit de l'étape (1) pour déterminer la présence ou l'absence du polymorphisme.

4. Méthode de la revendication 3, dans laquelle l'endonucléase de restriction thermostable reconnaît l'acide nucléique ne comprenant pas le polymorphisme.

5. Méthode selon la revendication 2 ou la revendication 4 dans laquelle la méthode comprend de plus les étapes additionnelles suivantes :

(3) faire réagir l'acide nucléique amplifié de l'étape (1) avec au moins une endonucléase de restriction, la au moins une endonucléase de restriction étant sélectionnée de façon qu'elle digère l'acide nucléique amplifié comprenant un polymorphisme particulier ; et
(4) déterminer si la digestion se produit à l'étape (3), la digestion indiquant la présence du polymorphisme particulier.

6. Méthode selon l'une quelconque des revendications 1 à 5 dans laquelle le procédé impliquant le thermocycle est PCR.

7. Méthode selon l'une quelconque des revendications 1 à 6 dans laquelle l'analyse de l'étape (2) comprend la détection de la présence ou de l'absence de l'acide nucléique amplifié de l'étape (1), la présence ou l'absence de l'acide nucléique amplifié indiquant la présence ou l'absence du polymorphisme.

8. Méthode selon l'une quelconque des revendications 1 à 7 dans laquelle l'acide nucléique est l'ADN.

9. Méthode selon l'une quelconque des revendications 1 à 8 dans laquelle l'endonucléase de restriction thermostable est sélectionnée dans le groupe consistant en *Bst* NI, *Bsl* I, *Tru* 91 et *Tsp* 509 I.

10. Méthode selon l'une quelconque des revendications 1 à 9 dans laquelle le polymorphisme génétique est détecté dans l'un des proto-oncogènes *ras*, K-*ras*, N-*ras* et H-*ras*, ou le gène suppresseur de tumeur p53.

11. Méthode selon la revendication 10 dans laquelle le polymorphisme génétique est détecté dans le codon 12 de K-*ras*.

12. Méthode selon l'une quelconque des revendications 1 à 9 dans laquelle le polymorphisme génétique est détecté dans HIV-I, le régulateur de conductance trans-membrane de la fibrose kystique, l'α-antitrypsine ou la β-globine.

13. Méthode selon la revendication 9 dans laquelle l'endonucléase de restriction est *Bst* NI et l'amplification de l'acide nucléique se produit en présence d'un tampon comprenant au moins NaCl environ 50mM et au moins $MgCl_2$ environ 3mM.

14. Méthode selon la revendication 13 dans laquelle la concentration en NaCl est d'environ 100mM.

15. Méthode selon la revendication 13 dans laquelle la concentration en $MgCl_2$ est de 3 à 10mM.

16. Méthode selon la revendication 9 dans laquelle l'endonucléase de restriction est *Bsl* I et l'amplification de l'acide

nucléique se produit en présence d'un tampon comprenant DTT.

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Real time detection of DNA·RNA hybridization in living cells

(molecular beacon/antisense/oligodeoxynucleotide/fluorescence resonance energy transfer)

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ABSTRACT **Demonstrating hybridization between an antisense oligodeoxynucleotide and its mRNA target has proven to be extremely difficult in living cells. To address this fundamental problem in antisense research, we synthesized "molecular beacon" (MB) reporter oligodeoxynucleotides with matched fluorescent donor and acceptor chromophores on their 5′ and 3′ ends. In the absence of a complementary nucleic acid strand, the MB remains in a stem–loop conformation where fluorescence resonance energy transfer prevents signal emission. On hybridization with a complementary sequence, the stem–loop structure opens increasing the physical distance between the donor and acceptor moieties thereby reducing fluorescence resonance energy transfer and allowing a detectable signal to be emitted when the beacon is excited by light of the appropriate wavelength. Solution hybridization studies revealed that in the presence of a complementary strand targeted MB could yield up to a 60-fold increase in fluorescence intensity in comparison to control MB. By using a fluorescence microscope fitted with UV fluoride lenses, the detection limit of preformed MB/target sequence duplexes microinjected into cells was found to be $\geq 1 \times 10^{-1}$ ag of MB, or ~10 molecules of mRNA. On the basis of this exquisite sensitivity, real-time detection of MB/target mRNA hybridization in living cells was attempted by microinjecting MB targeted to the *vav* protooncogene, or control MB, into K562 human leukemia cells. Within 15 min, confocal microscopy revealed fluorescence in cells injected with targeted, but not control, MB. These studies suggest that real-time visualization and localization of oligonucleotide/mRNA interactions is now possible. MB could find utility in studying RNA processing, trafficking, and folding in living cells. We hypothesize that MB may also prove useful for finding targetable mRNA sequence under physiologic conditions.**

Antisense oligodeoxynucleotides (AS ODNs) are being evaluated for treatment efficacy of viral (1), cardiovascular (2, 3), gastrointestinal (4, 5), and neoplastic diseases (6–8). The ODNs are thought to perturb gene expression by hybridizing with their complementary mRNA, thereby promoting translation arrest or, perhaps more likely, physical destruction of the mRNA by an RNase H-dependent mechanism (9–11). Nevertheless, studies that provide direct physical evidence for ODN/mRNA hybridization *in vivo* are rare. Decrement in targeted mRNA levels (12–14) or down-regulation of targeted protein (15–18) is widely used to infer duplex formation but RNA fragments consistent with ODN-guided cleavage have been described only in *Xenopus* oocytes (19) and hematopoietic cells (10). Whether such fragments truly represent the result of hybridization in the living cell is uncertain. Because it is widely appreciated that ODN may effect a multitude of cellular process through mechanisms that do not involve hybridization with a specific mRNA (20, 21), this issue is clearly at the core of all "antisense" research.

To demonstrate ODN/mRNA hybridization *in vivo*, we were attracted to methods using fluorescence resonance energy transfer (FRET) (22, 23). The principle of FRET is based on transfer of electronic excitation energy through dipole–dipole coupling of a fluorescent donor to an acceptor. Transfer efficiency depends on donor and acceptor transition dipole orientation, distance between the molecules, and spectral properties of the donor and acceptor. FRET has been used to detect duplex formation between complementary ODN in solution (24–28) and within living cells (29). However, a potential problem with FRET-detected hybridization is that other phenomena may lead to loss of cellular fluorescence. Therefore, in a living cell one cannot be certain that loss of signal means conclusively that hybridization has actually taken place. The "molecular beacon" (MB) concept of Tyagi and Kramer (30, 31) addresses this concern. These molecules have a stem–loop structure with fluorophore and quenching moieties annealed to the 5′ and 3′ ends of the molecule, respectively (Fig. 1). The fluorophore does not modify the structure of the hairpin (32) thereby permitting the loop sequence to hybridize with its respective complementary nucleic acid sequence. Once duplex formation occurs, the fluorophore and quencher become separated in space and FRET is no longer possible. The fluorophore will then emit light of the appropriate wavelength when excited. This event is readily detected in solution by use of a fluorimeter or by direct observation using a suitably equipped microscope. We demonstrate herein that MBs can be used to directly demonstrate duplex formation in living cells.

MATERIALS AND METHODS

Cell Lines. TK⁻ts13 (hamster fibroblasts), and K562 human leukemia cells were obtained from the American Type Culture Collection.

Synthesis of MBs. MBs were synthesized as described (30) and are illustrated in Fig. 1. The 5′ fluorophore donor used was EDANS [5-(2′-aminoethyl)aminonapthalene-1-sulfonic acid]. DABCYL [4-(4′-dimethylaminophenylazo) benzoic acid] was used as the 3′ acceptor. "Stem" structures were formed by the same complementary 5′ (GCGAG) and 3′ (CTCGC) nucleotides. These flanked the following loop sequences: vav AS, GTTCTTAAGGCACAGGAACTGGGA; β-actin AS, CGCGGCGATATCATCATCCATAAC; vav sense control, TCCCAGTTCCTGTGCCTTAAGAAC; β-actin sense con-

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Abbreviations: AS, antisense; ODN, oligonucleotide; MB, molecular beacon; FRET, fluorescence resonance energy transfer; EDANS, 5-(2′-aminoethyl)aminonapthalene-1-sulfonic acid; DABCYL, 4-(4′-dimethylaminophenylazo) benzoic acid; SCR, scrambled.

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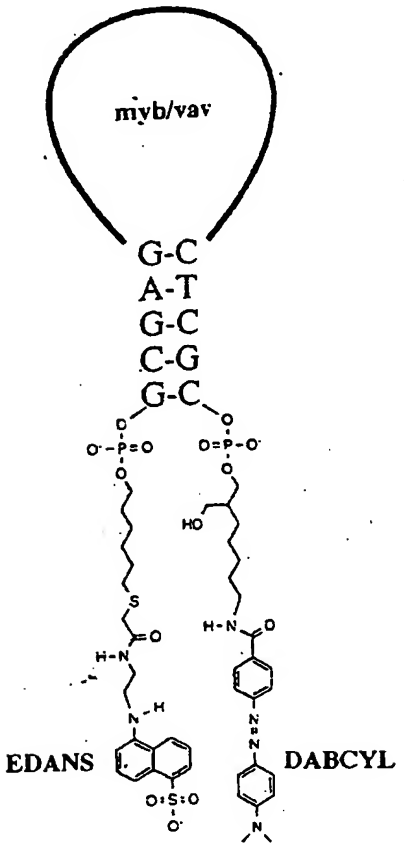

FIG. 1. MB structure. MBs were synthesized with a 24-nt "loop" sequence flanked on the 5' and 3' ends with complementary sequence 5 nt long. Internal hybridization of the complementary ends creates the stem–loop structure. The fluorescent donor (EDANS) and acceptor (DABCYL) molecules are joined to the 5' terminal phosphate and the 3' terminal hydroxyl group, respectively, through (CH2)6-S-CH2-CO and (CH2)7-NH linker arms.

trol, GTTATGGATGATGATATCGCCGCG; vav Scrambled (SCR) control, AGACCTGGTTGTTCACGGGAAAAG; β-actin SCR control, GGTAGATGCAGCCTTGTCTATACC; vav mismatch control, GTTCTTAACCGTGTCGAACTGGGA; β-actin mismatch control, CGCGGCGATAGGTACATCCATAAC.

Fluorescence Detection of Hybrid Formation. AS or control MB (final concentration, 150–200 nM) dissolved in MB buffer (100 mM Tris·HCl, pH 8/1 mM $MgCl_2$) was added to an excess of complementary target ODN (20–30 μM). Fluorescence emission was monitored in a Hitachi/Perkin–Elmer MPF4 spectrofluorimeter. Controls were composed of a 6-bp mismatch, SCR, or sense (SEN) loop sequences. The excitation wavelength of EDANS was 336 nm, and the emission maximum was 490 nm. The efficiency of FRET between the donor and acceptor was recorded by scanning the emission spectra from 375 nm to 525 nm for the EDANS/DABCYL pair after excitation at 336 nm in a fluorimeter. In all experiments, the background fluorescence intensity of the MB buffer was negligible as was the background fluorescence of described controls when sense target sequences were added to the solution. Each MB was evaluated for fluorescence emission after addition of its complementary target sequence.

Electrophoretic Analysis. Samples containing AS-MB/sense ODN duplexes, as well as control MB with this same template, were analyzed on nondenaturing 8% polyacrylamide gels. After electrophoresis of samples at 70 V for 1.5 h, gels were soaked in a solution of ethidium bromide at 50 μg/ml and examined on a UV light box. The mobility of duplexed material was retarded on these gels in comparison to unduplexed material.

DNase I Action. A sample containing unhybridized MB was equilibrated at 37°C and then 5 μl of DNase 1 (Boehringer Mannheim) enzyme was added in DNase I buffer (400 mM Tris·HCl, pH 7.4/100 mM NaCl/60 mM $MgCl_2$/100 mM $CaCl_2$). The fluorescence emission of these solutions was monitored through fluorimetric scans as described above.

Hybridization to Total RNA. Total RNA was extracted from hematopoietic K562 cells as described (33). RNA (3 μg) was heated to 95°C for 5 min and then hybridized to AS or control MBs. After hybridization overnight at 37°C, 0.6 ml of Tris buffer containing 1 mM $Mg Cl_2$ was added, and samples were centrifuged for 20 min to remove particulate matter. The scanning emission spectra in a fluorimeter was completed as described.

Microinjection of MBs and Preformed Duplexes. K562 cells were centrifuged at 300 rpm for 10 min onto cytospin slides that had been treated with CELL-TAK (Collaborative Bio-Medical Products, Bedford, MA). Rings were immediately drawn with a hydrophobic PAP pen (Kiyota International, Elk Grove Village, IL) and RPMI 1640 medium supplemented with 10% bovine calf serum was dropped into the ring to ensure survival of the cells. Slides were viewed under an IMT-2 inverted microscope while performing microinjection. Approximately 10–100 pl of MBs, resuspended in MB buffer, was injected per cell. Approximately 100–200 microinjections were completed within 10 min. Slides were coverslipped and immediately exposed to UV light for 50 s. Images were grabbed with a KS-1381 Videoscope (Videoscope International, Washington, DC) and fluorescence levels were measured by using CUE series image analysis software, with reference to uniformly dyed microspheres of 19.5 μM diameter (product LF97371/171–1, Bangs Laboratories, Carmel, IN).

Alternatively to grabbing images, 35-mm slide film was exposed to the images with an Olympus C-35AD-4 35-mm camera with an attached exposure control unit set on automatic. Photographic images were also captured at various time intervals, at different exposure settings that were then recorded.

Confocal Laser Scanning Microscopy. A Leitz confocal laser scanning module attached to an inverted microscope was used to monitor fluorescence emission. This microscope was equipped with a broad-range UV lens that stimulates at a wavelength of 351 nm. To optimize the UV fluorescence emission, the 440–40 long-pass filter was set to channel 1. The long-pass 450 filter was set to channel 2, and the substrate was set to dichroic so that all fluorescent energy was sent to channel 2. To prevent photobleaching, the confocal microscope was operated under conservative conditions (low illumination and intermediate slit positions., 14–25 μm, to maximize signal strength).

RESULTS

MB Design. A 34-nt MB stem–loop structure composed of 5-nt complementary flanking sequences and a 24-nt intervening loop sequence was synthesized (Fig. 1). EDANS, the fluorescent donor, and DABCYL, the acceptor, were conjugated to the 5' and 3' ends of the molecule, respectively, through spacer arms. The major nucleic acid sequence constraints for MB design have been reported (30). They presume that the molecule assumes a stem–loop structure in the absence of a complementary sequence. In the stem–loop configuration, the EDANS and DABCYL moieties are in close enough proximity so that FRET occurs and no fluorescent signal is observed. In the presence of a complementary target sequence, a bimolecular helix is formed causing the stem–loop

structure to open. The fluorophore and quenching moieties then move far enough apart in space so that FRET is diminished or nonexistent. A detectable signal may then be emitted upon excitation of the fluorescence donor group on the MB molecule.

To test these predictions, MBs with different loop sequences were mixed in solution with a variety of ODN target sequences, excited by UV light (336-nm peak wavelength), and then examined by a spectrofluorimeter for the emission signal. Sequences used were given above. As expected, fluorescence signal was detected only when MBs were placed in solution with the ODN target to which they could hybridize (Fig. 2). For example, when an ~137-fold molar excess of a target ODN corresponding to vav nucleotides 195–218, or β-actin nucleotides 8–31, was incubated at room temperature with MBs containing loops corresponding to either vav or β-actin AS, SCR, or mismatched (vav-7 and β-actin-4 nucleotides) sequence, fluorescence signal was observed only when the target ODNs and MBs were complementary to each other (Fig. 2 *A–D*). The specificity of these reactions is worth emphasizing. Signal above background was never detected when target sequence and MB were mismatched at four or more bases.

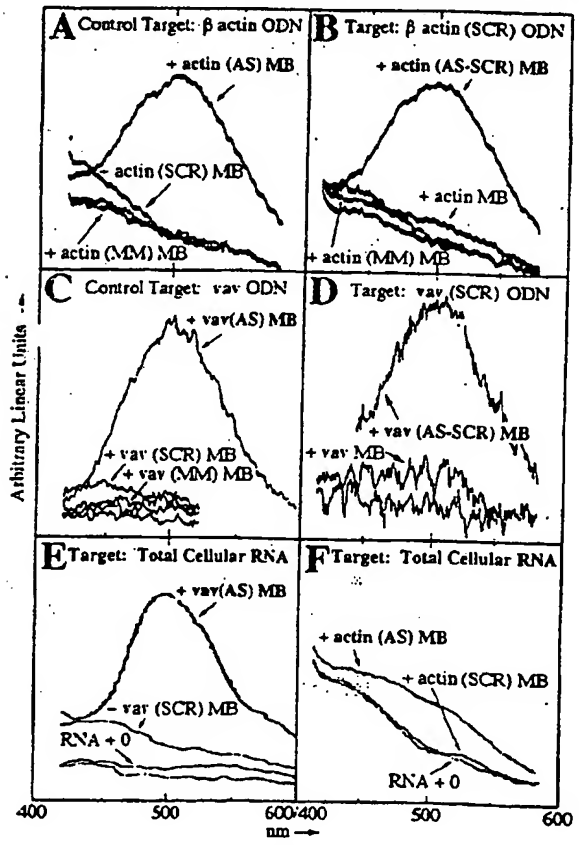

FIG. 2. Specificity of AS MB double helical and hybrid formation in solution. (*A–D*) Fluorescence emission spectra when various MB sequences were added to solutions of target ODNs. Hybridization was detected by spectrofluorimetry. Excitation wavelength was 336 nm. Signal intensity is displayed along the vertical axis, and the wavelength scanned is displayed on the horizontal axis. An increase in fluorescence intensity, ranging from 15- to 60-fold in comparison to background, was only observed with complementary MB/oligonucleotide pairs. Lowest baselines are tracings derived from solutions containing buffer or ODNs only. (*E* and *F*) Spectrofluorimetric tracings derived from AS-MB or SCR-MB, no MB, or MB buffer only incubated with total RNA extracted from K562 hematopoietic cell lines. Addition of MBs complementary to vav (*E*) or β-actin (*F*) mRNA sequence resulted in 15- and 9-fold increases in fluorescence intensity respectively in comparison to controls.

Interestingly, fluorescence signal intensity varied with the target/MB pair used (Fig. 2 *A–D*). For vav target/MB duplexes, for example, a signal ~30 times greater than background was observed, a result consistent with beacon fluorescence intensity reported by Tyagi and Kramer (30, 31). In contrast, β-actin/MB duplexes generated signal only ~15 times greater than background. These differences might be explained by the nature of the intramolecular folding and/or hybridization permitted by the MB sequence used. Such folding could impact on hybridization efficiency with consequent effects on output signal. It is important to note that when MB sequences were digested with DNase I, full fluorescence emission was observed in the fluorimeter (data not shown). Accordingly, the results in aggregate strongly suggest that the predicted stem–loop structures were in fact forming and that, when DNA sequence complementary to the MB loop structure was added to the reaction, the anticipated hybridization induced conformational change took place, leading to fluorescence detection in an appropriately excited sample.

We also examined the ability of the various MBs to hybridize with target in a pool of total cellular RNA. RNA was extracted from K562 cells and then mixed with AS sequence MBs or control nucleic acid sequence MBs. In comparison to control MB, we observed a 15-fold increase in fluorescence signal when AS-*vav*-MBs were allowed to hybridize with total K562 RNA (Fig. 2*E*). AS-β-actin MB showed a 9-fold rise in fluorescence emission in comparison to its controls (Fig. 2*F*), a result in accord with the data presented above.

Detection of Endogenous MB/mRNA Hybridization. By using serial dilutions of preformed MB/ODN duplexes microinjected into K562 cells, we found that we could detect as little as 1×10^{-1} ag of material, which corresponds to ~10 molecules of AS vav MB. If the average cell is 10^{-12}–10^{-11} liters, we calculate that we can detect 20–200 pM RNA. On the basis of these calculations, we hypothesized that we would be able to detect MB/mRNA hybridization, real time, in living cells. We tested this prediction by microinjecting 150 μM *vav* AS or SCR (control) MBs into living K562 human leukemia cells. After injection, the cells were examined for signal by phase and fluorescence microscopy (Fig. 3). Higher levels of cellular fluorescence were observed in cells injected with AS MB (Fig. 3 *C* and *D*) than in those cells injected with SCR MBs (Fig. 3 *A* and *B*). Uninjected cells displayed no discernible fluorescence (Fig. 3 *E* and *F*). Specificity of the fluorescence signal was further determined by injecting fibroblasts with AS MBs or vav(SCR) MBs. Both were comparable in fluorescence with vav SCR MB-injected K562 controls. In marked contrast however, cells injected with β-actin AS MBs had a fluorescence signal that was twice the intensity of the control cells (data not shown).

We quantified the intensity of fluorescent signals emitted from MB/mRNA duplexes by using CUE series image analysis software. Fluorescence intensity of AS-*vav*-MB complexed to target mRNA was approximately 2.2-fold higher than sequence matched controls relative to a manufactured fluorescent standard (Bangs Laboratories; Fig. 4). The fluorescence intensity of AS-MB targeting β-actin sequences was approximately 5.5 fold higher than matched controls. This result was anticipated because β-actin mRNA is expressed at higher copy number than vav. Approximately 50 cells were injected with either AS or control MB and examined for fluorescence emission within each experiment. The experiment was repeated three times and data are reported as the mean ± SD.

We then attempted to more finely localize the intracellular location(s) of MB/mRNA duplex formation by using confocal microscopy. AS and control MBs were injected into living cells which were then examined as described in the methods (Fig. 5).

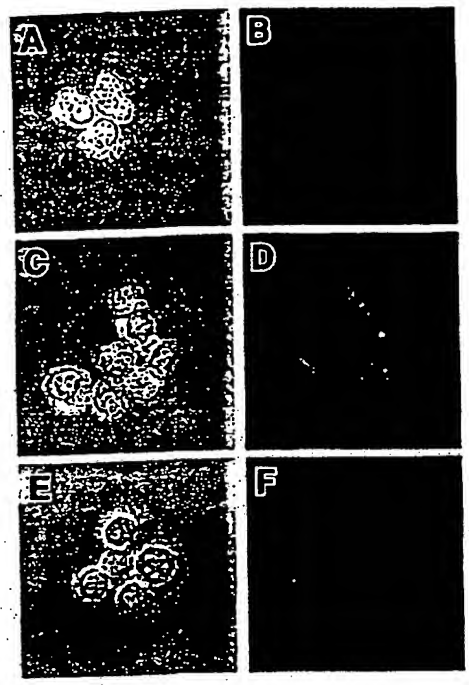

FIG. 3. Fluorescence emission of MB after microinjection into K562 hematopoietic cells. After injection of 150 μM vav AS or SCR (control) MBs into living K562 human leukemia cells, the cells were examined for signal by phase (*A*, *C*, and *E*) and corresponding fluorescence (*B*, *D*, and *F*) microscopy. Significantly higher levels of cellular fluorescence were observed in cells injected with AS MBs (*C* and *D*) than in those cells injected with SCR MBs (*A* and *B*). Uninjected cells displayed no fluorescence (*E* and *F*). *A* shows uninjected control cells photographed under phase microscopy; *B* is the corresponding fluorescent photomicrograph. *C* and *E* are AS-MB- and SCR-MB-injected K562 cells, respectively, photographed under phase microscopy, and *D* and *F* are their corresponding fluorescent counterparts, respectively. Note that maximal fluorescent emission is found in AS-MB-injected cells.

Little or no signal was observed in cells injected with control (SCR sequence) MB (data not shown). In contrast, cells injected with AS vav MBs revealed fluorescence signal when irradiated by a laser tuned to excite at 351 nm (Fig. 5). Considerably more fluorescence was observed in the area corresponding to the cell's cytoplasm than nucleus, suggesting that hybridization was favored in the latter location.

Speed and Integrity of AS-MB/mRNA Duplex Formation *in Vivo*. To gain an appreciation of the time course of hybridization between vav targeted AS MBs and their mRNA targets, we microinjected MBs into ~100 K562 cells under direct microscopic visualization by using a Narshige micromanipulator (Medical Systems, Greenvale, NY). All injections were accomplished within ~14 min after which the slides were coverslipped and illuminated with UV light for 50 s. The slides were then viewed with a fluorescence microscope equipped with UV fluoride lenses. Fluorescence was easily detectable in the AS-MB-injected cells although none was observed in cells injected with control MBs. Accordingly, these experiments indicate that the injected MBs find and hybridize with their endogenous mRNA target within 15 min of being introduced into the cell.

In additional experiments we noted that control MBs exposed to constant UV light also emitted fluorescent signals after approximately 45 min of excitation. We noted, however, that control MB exposed to constant UV light also emitted fluorescent signals after approximately 45 min of excitation. This observation suggests that intracellular nucleases may have degraded the MB hairpin-loop structure or that transient opening or "breathing" of the MB molecule was occurring. Either explanation is possible because fluorescence would not be observed in MBs that maintained their predicted conformation. Nonetheless, the apparent degradation of MB/target duplexes over time can be used to estimate the half-life of the MBs in an intracellular environment. From examination of more than 800 cells within eight experiments, phosphodiester MBs appear to undergo significant hydrolysis after ~45 min.

DISCUSSION

The goal of the studies described herein was to determine whether the MB strategy could be used to identify and quantify intracellular duplex formation between physiologically relevant mRNA species and appropriately targeted MB. For this purpose we synthesized MB with 5' fluorescent EDANS groups and a DABCYL quencher coupled to the 3' end. Loop sequences were designed to hybridize with endogenous vav mRNA, which encodes an important hematopoietic cell signaling protein (34), and the highly expressed housekeeping gene β-actin. The results of our studies demonstrate clearly that MB may be used to detect hybridization events *in vitro*. In solution, for example, we observed up to a 60-fold increase in fluorescence emission intensity when MBs hybridized to complementary ODNs in solution. In a more relevant model, up to a 15-fold increases in fluorescence intensity, in comparison to controls, was observed when MBs were allowed to hybridize with complementary sequences found in the total RNA fraction of K562 human leukemia cells. These results suggested that even in a large pool of competing mRNA species, specific detectable hybridization could take place.

The *in vitro* results described above further suggested the possibility of using MBs to monitor mRNA hybridization, real time, in living cells. We examined this possibility in an orderly fashion. First, preformed hybrids were microinjected into K562 cells to determine the possibility of detecting scarce to intermediate frequency target mRNA molecules (15–300 copies) within the cell. We found we could detect fluorescence signal from such complexes after injection of as little as 1×10^{-1} ag of material, or ~10 molecules of MB. Next, we microinjected 150 μM vav targeted AS or control MB into the nucleus of K562 cells. Cells injected with the AS molecule emitted detectable fluorescence ~2- to 5.5-fold higher than controls within 15 min. Specificity of these reactions was demonstrated in a number of ways including the use of numerous control MB injections, and injection of AS MBs into cells which do not express the targeted message. In all cases, signal clearly above background was observed only under those conditions in which it was expected, i.e., when the MB and its targeted complement were present in the cell under study.

The speed, and apparent integrity, of duplex formation was assessed by examining cells for fluorescence at specific time periods after microinjection. Hybridization appeared to occur within 15 min of introducing MB into the nucleus of K562 cells. Little or no detectable fluorescence was observed in cells injected with control MB at similar observation points. Detailed kinetics of hybridization within this time period are presently being examined. However, after ~45 min, fluorescence was observed in cells injected with control MBs. While MBs may be slightly more resistant to nucleases than linear ODNs because of the presence of the Mg-stabilized 5',3'-derivatized stem structure (30), we speculate that the appearance of fluorescence in control injected cells is likely the result of MB degradation. Alternatively, binding of proteins that destabilize the hairpin structure and cause it to open may also play a role in this effect (35–37).

MBs may be more fully stabilized by chemical modifications to the internucleotide linkages, which would provide greater nuclease resistance (38–40). In addition to modifications to

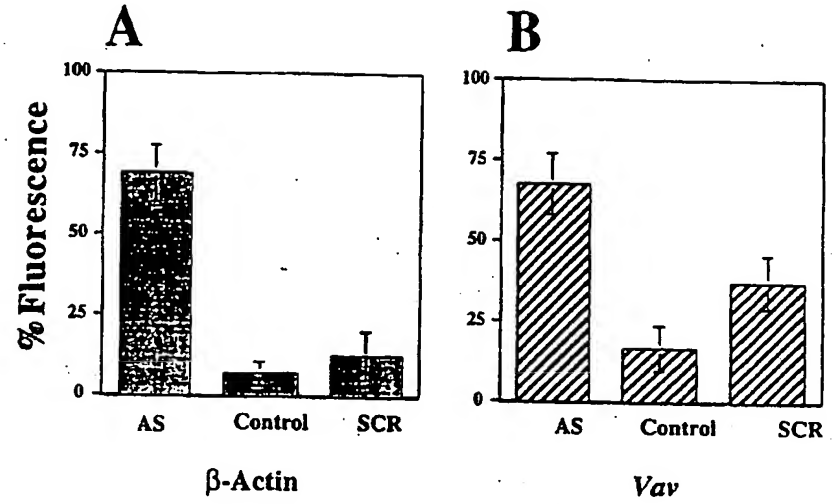

FIG. 4. Relative fluorescence intensity of molecular beacons. K562 cells were microinjected with AS-, SCR-, or NO-β-actin (*A*) or *vav* (*B*) MBs at 150 μM. MBs hybridized to target sequences within 14 min. Cells were then exposed to UV light by using a ×60 plan Apo numerical aperture 1.4 UV fluoride lens. Fluorescent signals were observed within 50 s, and images were captured with a KS-1381 videoscope. Fluorescence levels were measured by using CUE series image analysis software with reference to uniformly dyed microspheres.

the internucleoside bridge, sugar or base alterations may also be expected to lend increased stability to MBs (41–43). More stabilized molecules could reasonably be expected to generate higher signal to noise ratios. Higher levels of fluorescence might also be obtainable by redesigning loop sequence with impaired ability to undergo intramolecular folding. Alternatively, because mRNA associated proteins and/or tertiary structure may govern the ability of MBs to hybridize with their target, targeting different portions of the mRNA molecule might also be considered (8, 44). MB stabilization and higher signal to noise ratio would clearly be useful for more mechanistic studies on ODN sorting within cells and defining locations where duplex formation occurs most readily. We further speculate that MBs might prove useful for finding accessible regions within an RNA molecule that one wished to target for destruction with an antisense oligonucleotide strategy.

FIG. 5. Confocal image of K562 cells injected with AS vav MBs. Cells injected with AS vav MBs revealed fluorescence signal when irradiated by a laser tuned to excite at 351 nm. Fluorescence images were gathered 15–30 min after MB injection and appeared stronger in the cells' cytoplasm (outlined by green arrows) than in the nucleus (white arrow), suggesting that hybridization may be favored in the latter location. Uninjected cells or cells injected with control MBs displayed little or no signal and were, therefore, very dark or unseen.

In addition to direct visualization of ODN/mRNA duplex formation, stabilized MBs with strongly emitting fluorophore moieties may lend insight into the mechanism of action of AS-ODNs within cells. For example, when target mRNA is processed in the nucleus and exported to the cytoplasm, an MB duplexed with the mRNA should allow this transition to be monitored real time by confocal fluorescence microscopy. If RNase H-assisted catalysis occurs in the nucleus, MB fluorescence would be lost in this location because of reformation of stem–loop sequences. The nuclei of effected cells would, therefore, appear dark. Alternatively, if RNase H-mediated degradation of the mRNA duplex does not occur, and the MB/mRNA hybrid is transported to the cytoplasm, it should be possible to demonstrate complex colocalization with ribosomes and possibly interference with translation if fluorescent signals remain in the same intracellular location. Other uses for the MBs might easily be contemplated including their use to study mRNA folding and processing, *in vivo*. MB might also act as reporters of viral invasion or the presence of mutated mRNA transcripts *in vivo*. With optimization of MB stability and fluorescence emission, these molecules should become valuable tools for studying many aspects of RNA biology in living cells.

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